昭和32年7月9日 第三種郵便物認可
第46巻 第8号 昭和34年8月25日発行
(毎月1回25日発行)
The Journal of Biochemistry, published monthly by the Japanese Biochemical Society, Tokyo, Japan.
Vol. 46, No. 8, August 25, 1959

Vol. 46, No. 8 **August, 1959**

THE JOURNAL OF BIOCHEMISTRY

EDITED FOR THE JAPANESE BIOCHEMICAL SOCIETY

PUBLISHED MONTHLY

BY

THE JAPANESE BIOCHEMICAL SOCIETY

Tokyo University, Tokyo

AGENCIES

Charles E. Tuttle
Rutlant, Vt.
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Maruzen Co., Ltd.
Tokyo
Japan

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Köln
Germany

PRINTED IN JAPAN

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STUDIES ON THE METHYLATION OF PYRIDINE COMPOUND IN ANIMAL ORGANISMS

III. THE METHYLATION PATTERN OF PYRIDINE IN DOG ORGANISMS DOSED WITH PYRIDINE*

By YOSHIO OKUDA

(*From the Biological Institute of Kobe University, Kobe*)

(Received for publication, May 30, 1958)

An animal organism is more or less able to accomplish *N*-methylation of the pyridine-ring of pyridine derivatives, as is the case with pyridine itself. When pyridine is given to animal organisms, a part is excreted unchanged, whereas a part undergoes *N*-methylation giving *N*-methylpyridine (Methyl-pyridyl-ammonium-hydroxide), although the extent varies according to animal species. In the case of dog organisms it is known that methylation of pyridine readily takes place (*1*, *2*).

The present paper deals with how the methylation pattern of pyridine in dog organisms is formed, by examining the rise and fall of *N*-methylpyridine excreted in the urine of dog organisms various hours after receiving pyridine, either by injection or per os, since *N*-methylpyridine is a resultant product by methylation of pyridine.

EXPERIMENTALS AND RESULTS

(*I*) *Materials and Methods*—A 7 month old female dog was used throughout the experiment. For the detection and separation of methylated pyridine in samples paper chromatography is used, and the quantitative estimation is made by measuring the optical density at ultra-violet region using Shimazu Q. S. 20 photoelectric spectrophotometer.

(*II*) *Preparation of Samples*—Series I: Various hours after dosing, the urine was collected from the dog receiving 0.5 g. of pyridine and immediately evaporated in vacuo at 55–60° to a syrup. Each residue is extracted with ethanol and filtrated. The filtrate is then evaporated, and the residue is dissolved in 5 ml. of acetone and was used as samples.

Series II: The urine is also obtained from the same dog receiving 0.5 g. of pyridine by subcutaneous injection, and treated in the same manner as that of series I.

The relationship between the materials and the samples of two series is given in Table I.

* Contributions from the Laboratory of Biological Institute, Kobe University, Kobe, No. 57.

TABLE I

Time of the Day When the Urine Is Collected for the Test Samples

Date (Sept. 1957)	Time of the day	Sample No.	Quantity of urine (*ml.*)	Hours after dosing (*hrs.*)
10th	6 p.m.	1	30	2
	10 ,,	2	50	6
11th	6 a.m.	3	110	14
	9 ,,	4	50	17
	12 p.m.	5	30	20
	5 ,,	6	60	25
	8 ,,	7	70	28
12th	6 a.m.	8	110	38
	10 ,,	9	40	42
	2 p.m.	10	35	46
	5 ,,	11	70	49
0.5 g. of pyridine is given to the dog by *per os* at 4 p.m. September 10, 1957.				
13th	9 p.m.	1	8	5
14th	4 a.m.	2	100	12
	7 ,,	3	20	15
	10 ,,	4	75	18
	1 p.m.	5	50	21
	5 ,,	6	50	25
	8 ,,	7	60	28
	11 ,,	8	80	31
15th	6 a.m.	9	120	38
	9 ,,	10	30	41
	12 p.m.	11	50	44

0.5 g. of pyridine is given to the same dog by subcutaneous injection at 4 p.m., September 13, 1957.

(*III*) *Detection and Quantitative Estimation*—For qualitative analysis, Toyo filter paper No. 51 single type is used, and 0.02 ml. of the sample solution is applied with a micro-pipette and developed with a following solvent system; *n*-butanol : acetic acid : water = 4 : 1 : 2 or 4 : 1 : 5 (by volumes). The chromatogram is dried, and sprayed with 2 per cent *p*-aminobenzoic acid alcohol solution (acidified with HCl). They are then exposed for 1 hour to BrCN vapor, whereby the *N*-methylpyridine appears as an obvious pinkish red spot, as shown in Fig. 1.

The spot was also found to be revealed by the fluorescence in the ultraviolet light.

The quantitative procedure is done by cutting out the section of the developed chromatogram which contains *N*-methylpyridine, eluting the

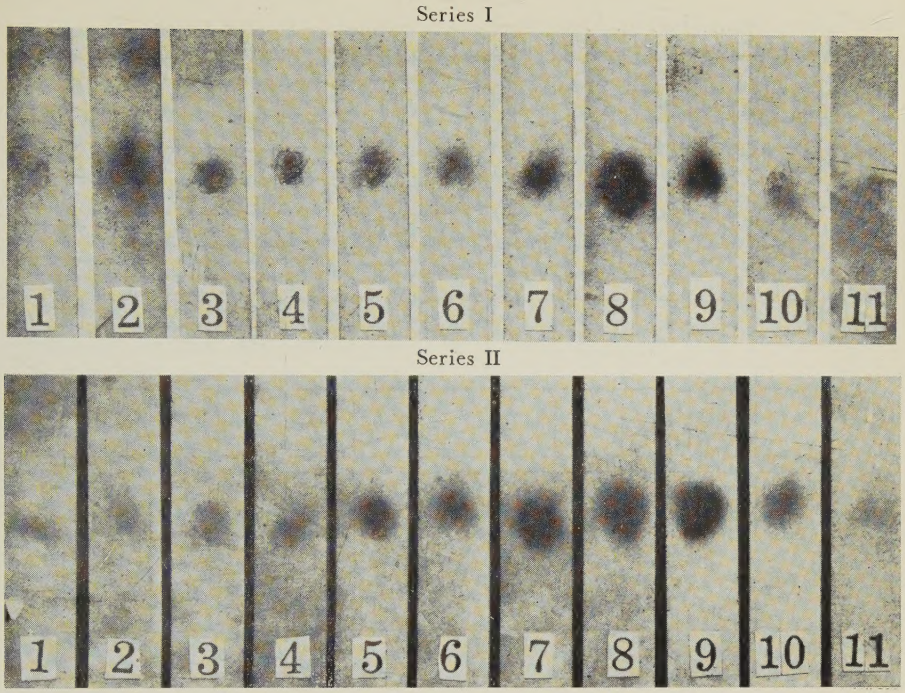

FIG. 1. The chromatograms of *N*-methylpyridine in urine of dog organisms dosed with 0.5 g. pyridine; Series I by *per os*, Series II by injection.

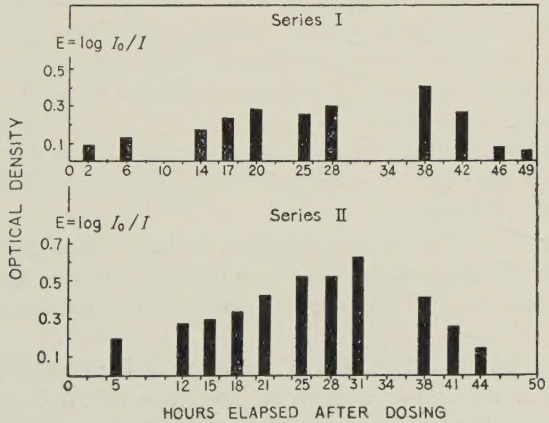

FIG. 2. Diagram showing the methylation pattern in dog organisms administered with 0.5 g. of pyridine; Series I by *per os*, Series II by injection. The height of each column denotes the optical density at ultra-violet region.

substances from the paper, and determining the amount in the eluate by measuring the optical density at 329 mμ with a 10 mm. absorption cell. The

results are shown in Fig. 2.

And the quantitative estimation of methylated pyridine in dog urine was made by measuring the optical density of ultraviolet region.

Thus the quantity of *N*-methylpyridine in the dog urine was estimated, as shown in Table II.

TABLE II

Quantitative Estimation of Methylated Pyridine in Dog Urine by the Optical Density at Ultraviolet Region

Hours after dosing	2	6	14	17	20	25	28	38	42	46	49	Total
Series I (*mg.*)	0.40	0.66	1.02	1.26	1.62	1.56	1.74	1.92	1.62	0.42	0.18	12.42

Hours after dosing	5	12	15	18	21	25	28	31	38	41	44	Total
Series II (*mg.*)	1.50	2.34	2.46	2.58	3.21	3.66	3.72	4.32	3.12	2.16	1.38	30.45

Series I by *per os*, Series II by injection.

DISCUSSION

As reported in the previous paper (*3*), inspection of the chromatograms reveals that both the intensity of color and the size of the spot vary with the quantity of the substance chromatographed, but the quantitative estimation is most suitably made by measuring the optical density at ultra-violet region by photoelectric spectrophotometer.

The separation of *N*-methylpyridine in the samples is therefore made by paper chromatography and its quantitative estimation was made by measuring the optical density at ultraviolet region.

The *N*-methylpyridine in samples is a resultant compound of methylation of pyridine, and the increase and decrease of this substance in urine collected various hours after dosing indicate the methylation pattern of pyridine in dog organisms.

In this case, the optical density of the samples is measured at the 329 mμ in wave length, a maximum absorption spectrum of *N*-methylpyridine, the optical density beeing proportional to the quantity of methylated pyridine.

SUMMARY

1. The methylation pattern of pyridine in dog organisms dosed with pyridine was studied. *N*-methylpyridine was separated by paper chromatography and determined by optical density at ultra-violet region using a

Beckman spectrophotometer.

2. In dog organisms, methylation of pyridine readily occurs and the resultant product, *N*-methylpyridir was excreted in the urine in 2 to 60 hours, the maximum being about 20 hours after dosing.

3. The total quantity of *N*-methylpyridine, excreted in the urine after pyridine injection was 2.5 times that after giving the same quantity of pyridine per os. Its methylation pattern in dog organisms is shown in Figs. 1 and 2.

4. When 0.5 g. of pyridine is given to dog organisms by subcutaneous injection or *per os*, *N*-methylpyridine is produced, the rise and fall of which various hours after dosing as is shown in Table II.

The author wishes to express his cordial thanks to Dr. M. Tomita for his kind encouragement and helpful criticism.

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STUDIES ON METABOLSIM OF AMIDES IN MYCOBACTERIACEAE

I. PURIFICATION AND PROPERTIES OF NICOTINAMIDASE FROM MYCOBACTERIUM AVIUM

By TOKUJI KIMURA

(From the Laboratory of Biochemistry, College of Science, St. Paul's University, Tokyo)

(Received for publication, October 21, 1958)

The hydrolysis of nicotinamide by micro-organisms has been described by Hughes and Williamson (*1*, *2*) in *Lactobacillus* and a strain of *Staphylococcus*, by Oka (*3*) in beer yeast and by Halpern and Grossowicz (*4*) in various species of *Mycobacteriaceae*.

This report will give the first information on the purified preparation of bacterial nicotinamidase: it is a study dealing with purification and properties of nicotinamidase from *Mycobacterium avium*.

METHODS AND MATERIALS

Quantitative Analysis—The rate of deamination of nicotinamide was determined by measuring the amount of ammonia formed. It was determined by Conway's micro-diffusion method. The protein concentration of the enzyme solution was measured by the biuret method.

Assay of Enzyme Activity—Standard assay mixture contained in 1.5 ml.: 16.6 μM of phosphate buffer (pH 8.3); 20 μM of nicotinamide and the enzyme (10–30 μg.). The reaction was started by the addition of the enzyme, and the incubation was carried out for 60 minutes at 38°. The blank control, which contained all components except the substrate, produced negligible amount of ammonia.

Definition of Unit and Specific Activity—One unit of activity was defined as the amount of the enzyme which gave 1.0 μM of ammonia under the above conditions. Specific activity was defined as units per mg. of protein.

Material Used—Nicotinamide, L-asparagine, L-glutamine, dihydrostreptomycin hydrochloride, and isonicotinic acid hydrazide were obtained from a commercial source.

RESULTS AND DISCUSSION

Purification of the Enzyme

Preparation of Acetone Powder of Mycobacterium avium—Acetone-dried cells of *Mycobacterium avium* (strain Takeo) grown in glycerol-bouillon medium were prepared as described previously (*5*).

Preparation of Purified Enzyme—Cell-free extract: 10 g. of the acetone-dried cells were finely ground with 60 g. of quartz sand and extracted by 300 ml. of

0.02 *N* phosphate buffer (pH 7.3). After standing over-night in a refrigerator, the cells were centrifuged off at 3,000 r.p.m. for 10 minutes, and the supernatant fluid was recentrifuged at 10,000 r.p.m. for 15 minutes at 0°.

Treatment with protamine sulfate: For the removal of nucleotides, 10 ml. of 1 per cent protamine sulfate were added to 280 ml. of the cell-free extract. After stirring, the mixture was allowed to stand for 30 minutes, then the precipitates formed were removed by centrifugation at 10,000 r.p.m. for 15 minutes. First ammonium sulfate fractionation: The supernatant fluid of protamine sulfate precipitation was brought to 30 per cent saturation by the addition of solid ammonium sulfate. After centrifugation, the precipitates were discarded and the supernatant fluid was next brought to 80 per cent saturation by the further addition of solid ammonium sulfate. The precipitated active material was centrifuged off, dissolved in a small amount of water and dialyzed against running water.

TABLE I

Summary of Purification of Nicotinamidase

Procedure	Nicotinamidase			Asparaginase
	Total protein (*mg.*)	Total units (*units*)	Specific activity (*unit/mg. protein*)	Specific activity (*unit/mg. protein*)
Crude extract	2380	5236	2.2	1.6
Protamine supernatant	1540	4004	2.6	—
1st ammonium sulfate fractionation	470	1880	4.0	1.4
2nd ammonium sulfate fractionation	118	14632*	124.0	2.5
Gel eluate	26	7410	285.0	—

* Refer the text.

Second ammonium sulfate fractionation: Solid ammonium sulfate was added to the above dialyzed solution of the enzyme. The fraction precipitating between 30 and 60 per cent saturation was collected. The precipitates were dissolved in 45 ml. of 0.01 *N* phosphate buffer (pH 6.0), and dialyzed against distilled water. After this procedure the total units of enzyme activity were remarkably increased due to the removal of inhibitors, probably metal ions. Calcium phosphate gel adsorption: 30 g. of calcium phosphate gel were added to the enzyme solution. After stirring, the suspension was allowed to stand for 30 minutes and centrifuged. The supernatant fluid had no activity, and the gel was washed once with 50 ml. of 0.01 *N* phosphate buffer (pH 6.0), and eluted 3 times, each with 40 ml. of 0.1 *N* phosphate buffer (pH 7.3).

Third ammonium sulfate fractionation: The gel-eluate was fractionated as before with ammonium sulfate. The fraction precipitating between 30 and

80 per cent saturation was collected. The precipitates, taken up in water, were dialyzed against distilled water. The enzyme preparations of the gel elution fraction were used throughout this experiment.

The summary of the purification procedure is shown in Table I. From the results obtained, it is suggested that *Mycobacterium avium* possessed at least L-asparaginase and nicotinamidase.

Properties of the Enzyme

Rate of Formation of Ammonia As a Function of Enzyme Concentration—The rate of formation of ammonia from nicotinamide is proportional to enzyme concentration as shown in Fig. 1.

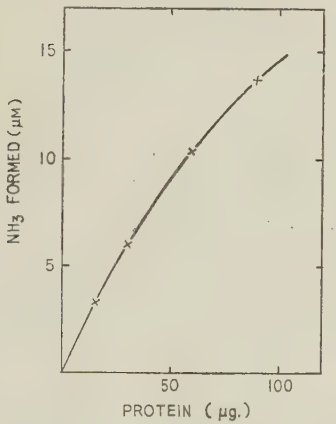

FIG. 1. Rate of formation of ammonia as a fuction of enzyme concentration.

Enzyme (gel eluate), as indicated; phosphate buffer (pH 8.3), 50/3 μM; nicotinamide, 40 μM; total volume, 1.5 ml.; 38°, 60 minutes.

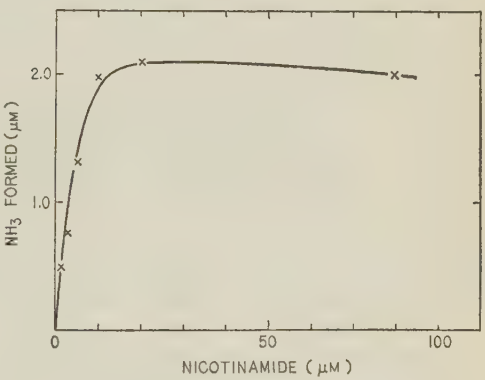

FIG. 2. Influence of substrate concentration.

Enzyme (gel eluate), 15 μg. of protein; phosphate buffer, (pH 8.3), 50/3 μM; nicotinamide as indicated; total volume, 1.5 ml.; 38°, 60 minutes.

Influence of Substrate Concentration—Fig. 2 shows the effect of the substrate concentration on the hydrolysis of nicotinamide. At the concentration of 13.3 μM per ml. the rate was almost maximum, and the further increase of the concentration reduced the rate slightly. From the data obtained, however, the Michaelis constant could not be calculated because the $1/V-1/S$ curve of Lineweaver-Burk plot did not show any linearity.

Effect of pH on Nicotinamidase Activity—The effect of pH on the nicotinamidase activity was studied. With the crude preparation, the enzyme was active over a wide range of pH values (7.0 8.3); however, with the most purified preparation, the optimum pH was 8.3. Tris-(hydroxymethyl)-aminomethane buffer showed inhibitive action (Fig. 3).

Stability of the Enzyme—When the purified enzyme was heated for 15

minutes at 50°, the enzyme activity was lost completely. When the crude enzyme preparations were frozen, the nicotinamidase lost approximately 13 per cent of its original activity, and 50 per cent of the asparaginase was destroyed. By dialysis also asparaginase was greatly damaged.

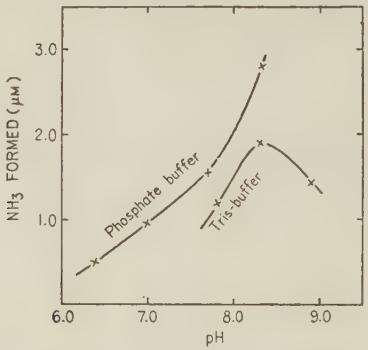

FIG. 3. Effect of pH on nicotinamidase activity. Enzyme (gel eluate), 15 μg. of protein; phosphate buffer, 50/3 μM or Tris-(hydroxylmethyl)-aminomethane, 25 μM; nicotinamide, 20 μM; total volume, 1.5 ml. 38°, 60 minutes.

Substrate Specificity—The most purified enzyme did not attack L-asparagine, L-glutamine, and DPN. However the crude extract could deaminate L-

TABLE II

Effect of Metal Ions on Nicotinamidase Activity

Experiment No.	Metal	(*M*)	NH_3 formed (μM)
I	None	—	0.71
	$MgCl_2$	3.3×10^{-4}	0.79
	$MnCl_2$	3.3×10^{-4}	0.66
	$CaCl_2$	3.3×10^{-4}	0.77
	$FeSO_4$	3.3×10^{-4}	0.63
	$CuSO_4$	3.3×10^{-4}	0.00
	$ZnCl_2$	3.3×10^{-4}	0.00
	$FeCl_3$	3.3×10^{-4}	0.00
II	None	—	1.57
	$CoCl_2$	3.3×10^{-4}	1.79
	$NiCl_2$	3.3×10^{-4}	1.89
	$CrCl_3$	3.3×10^{-4}	0.00

Enzyme (gel eluate), 10 μg. of protein in Experiment I, 20 μg. of protein in Experiment II, Tris buffer (pH 7.0), 25 μM; Nicotinamide, 20 μM; 38°, 60 minutes, Total volume, 1.5 ml.

asparagine, L-glutamine, and nicotinamide.

The Effect of metals and Inhibitors—The hydrolysis of nicotinamide by the purified enzyme was completely inhibited by such metal ions as Cu^{++}, Zn^{++}, Fe^{+++}, Cr^{+++} at 3.3×10^{-4} *M*, and Hg^{++} at 4.0×10^{-4} *M*. This enzyme was partially inhibited by metal-chelating agents. Therefore, it might be expected that the enzyme has a need for some metal ion; however, the enzyme activity was not affected by the addition of Mg^{++}, Mn^{++}, Ca^{++} Fe^{++}, Co^{++}, or Ni^{++} at 3.3×10^{-4} *M*. On the other hand, dihydrostreptomycin and isonicotinic acid hydrazide produced partial inhibition of the enzyme activity. (Tables II and III).

TABLE III

Effect of Inhibitors on Nicotinamidase Activity

Experiment No.	Inhibitor	(*M*)	NH_3 formed (μM)	Per cent of inhibition
I	None	—	3.65	—
	$HgCl_2$	4×10^{-3}	0.04	99
	EDTA*	4×10^{-3}	2.95	21
	o-Phen.	4×10^{-3}	1.85	50
	NR	4×10^{-3}	3.01	17
II	None	—	3.64	—
	SM	2×10^{-3}	2.70	26
	INAH	2×10^{-3}	2.10	41

Enzyme (gel eluate), 12.8 μg. of protein in Experiment I, 15 μg. of protein in Experiment II; phosphate buffer (pH 8.3), 50/3 μM; nicotinamide, 20 μM; total volume, 1.5 ml. 38°, 60 minutes.

* The following abbreviation is used: EDTA, ethylenediamine tetra-acetate; *o*-Phen., *o*-phenanthroline; NR. nitrose-R salt; SM, dihydrostreptomycin; INAH isonicotinic acid hydrazide.

SUMMARY

1. The enzyme which catalyzes the hydrolysis of nicotinamide has been purified about 250–280 fold from the extracts of *Mycobacterium avium* (strain Takeo). The purification procedures and properties of this enzyme are described.

2. The enzyme does not attack other amides, such as L-asparagine, L-glutamine, and diphosphopyridine nucleotide.

3. The enzyme activity is inhibited strongly by Cu^{++}, Zn^{++}, Hg^{++}, Fe^{+++} and Cr^{+++}, and inhibited moderately by metal chelating agents, dihydrostreptomycin, and isonicotinic acid hydrazide.

The author is indebted to Prof. T. Sasakawa for his interest and encouragement during the course of this study.

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STUDIES ON THE METABOLIC FUNCTION OF BIOTIN

IV. FUNCTION OF BIOTIN IN α-KETO ACID OXIDATION*

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(Received for publication, November 12, 1958)

In spite of the efforts of several workers (*1–5*) the mechanism of the retardation of the α-keto acid oxidation and the dehydrogenation reaction in the biotin-deficient organisms has not yet been elucidated. In 1951, however, Nason and his coworkers (*6*) discovered that the activity of the DPNase** had increased in the biotin-deficient *Neurospora*. This fact suggests the possibility that the retardation of the α-keto acid oxidation or the dehydrogenation reaction would be caused by the decrease of DPN.

Previously it was shown by the author (*7*) that when the rice-blast fungus, *Piricularia oryzae*, was cultivated under the biotin-deficient condition, pyruvic, α-ketoglutaric and dimethylpyruvic acids were accumulated in the culture medium and the contents of ATP as well as DPN in the mycelia were markedly decreased. The present investigation is concerned with the relation between the retardation of the keto acid oxidation and the decrease of DPN or ATP in the biotin-deficient cells of *B. macerans* and also the reasons for these phenomena.

EXPERIMENTAL

Cultivation of the Bacteria—The bacteria used in this experiment was *Bacillus macerans* Schardinger 3482 (ATCC 8518). The bacteria were grown in a modified Knight and Proom's medium (*8*) which contained the following nutrients per liter of distilled water; glucose 30 g., sodium glutamate 2.0 g., $(NH_4)_2HPO_4$ 7.0 g., KH_2PO_4 1.5 g., NaCl 1.0 g., $MgSO_4 \cdot 7H_2O$ 0.5 g., $CaCl_2 \cdot 2H_2O$ 0.3 g., $MnSO_4 \cdot 4H_2O$ 40 mg., $FeSO_4 \cdot 7H_2O$ 2.5 mg., ammonium molybdate 2.0 mg., thiamine hydrochloride 1.0 mg. and biotin. The amounts of biotin were 7.5 mμg. per liter for the biotin-deficient medium and 2,000 mμg. for the biotin-rich one respectively. The medium for subculture was the same as the biotin-deficient medium. After 40 hour cultivation the cells were collected by

* This work was presented in part before the Thirty-first Annual Meeting of the Japanese Biochemical Society, Sapporo, July 14–16, 1958.

** The usual abbreviations are used: ATP, ADP and AMP for the tri-, di-, and monophosphates of adenosine, DPN and DPNH for oxidized and reduced diphosphopyridine nucleotides, CoA for coenzyme A, DPNase for diphosphopyridine nucleotidase, ATPase for adenosine triphosphatase, TCA for trichloroacetic acid, Tris for tris(hydroxymethyl) aminomethane and DNP for 2,4-dinitrophenol respectively.

centrifugation and washed with 1/15 *M* phosphate buffer, pH 7.3, three times except Exp. 7,9 and 11 in which 0.09 per cent NaCl was used instead of phosphate buffer. The amount of cells used was determined from the transmittance at 660 mμ of the cell suspension in an appropriate buffer.

Analytical Methods—The activity of the keto acid oxidation was measured by use of the conventional Warburg manometer. For the determination of α-ketoglutaric acid, the author's salting-out extraction method of 2,4-dinitrophenylhydrazones (*9*) was used. Pyruvic acid was calculated by subtracting the value of α-ketoglutaric acid from that of the total keto acids. In all cases the keto acids accumulated in the biotin-deficient culture were identified as pyruvic acid and α-ketoglutaric acid by paper chromatography. The determinations of the amounts of coenzymes were carried out as follows: ATP by myosin method (*10*), CoA by Kaplan and Lipmann's method (*11*) and DPN by the cyanide addition method (*12*) and the method using alcohol dehydrogenase (*13*). For the determination of inorganic phosphate, the vanado-molybdate method (*14*) in Exp. 7 and 9, and Takahashi's method (*15*) or Yoda's method (*16*) in Exp. 11 were applied.

Materials—DPN was prepared by Yamada and others in this laboratory by Okunuki's method (*17*) and estimated to be 75 per cent pure. DPNH was prepared enzymatically (*18*) and its purity was proved to be 74 per cent. ATP was prepared in this laboratory by Berger's method (*19*) and had the purity more than 95 per cent. AMP was prepared by alkaline hydrolysis of ATP (*20*) and ADP was obtained from the Schwarz Laboratories, U.S.A. The hexokinase was purified from yeast by McDonald's method (*21*).

RESULTS

Keto Acid Accumulation (*Exp. 1, 2*)—After 40 hours cultivation the keto acids accumulated in the culture broth were analysed. The results are shown in Table I (Exp. 1). In this case most of the keto acids accumulated was proved to be pyruvate. The amount of pyruvate was observed to fluctuate considerably but the reason was not yet found. The accumulation of α-ketoglutarate was observed under more aerobic condition. After centrifugation and washing with phosphate buffer three times, the cells were suspended in 1/15 *M* phosphate buffer, pH 7.3, containing glucose as the substrate and aerated for 2 hours. As shown in Table I (Exp. 2) α-ketoglutarate besides pyruvate was observed to be accumulated in this experiment.

Oxidation of Keto Acid (*Exp. 3*)—While the biotin-rich resting cells were found to be able to oxidize glucose, pyruvate, α-ketoglutarate and dimethyl-pyruvate vigorously, the biotin-deficient cells weakly. In the case of glucose, the activity of oxidation was not so weak as in the case of pyruvate in the biotin-deficient cells. The results obtained when glucose and pyruvate were used as substrate are given in Fig. 1.

Activation of the Pyruvate Oxidation by Biotin (*Exp. 4*)—The pyruvate oxidation by the biotin-deficient cells was stimulated to some extent by preincubating the cells with biotin. More intensive stimulation was observed when the cells were preincubated with biotin and glucose (Fig. 2).

Effect of DPN and ATP on the Pyruvate Oxidation by Resting Cell (*Exp. 5*)—

Table I

Accumulation of α-Keto Acids in Media

Exp. 1: Analysis was carried out with the culture broth of the growing cells after cells were removed.

Exp. 2: The washed cells were suspended in 1/15 *M* phosphate buffer, pH 7.3, containing 3 per cent glucose and aerated for 2 hours at 37°. Then the cells were removed by centrifugation and the supernatants were analysed.

Exp. No.	Cell	Keto acid accumulated in media per 1 mg. of cells (μg.)	
		Pyruvic acid	α-Ketoglutaric acid
1	Biotin rich	2.12	trace
	Biotin deficient*	19.2 13600	trace
2	Biotin rich	20.0	trace
	Biotin deficient	135	21.0

* Among the results obtained, the minimum and the maximum-values were shown.

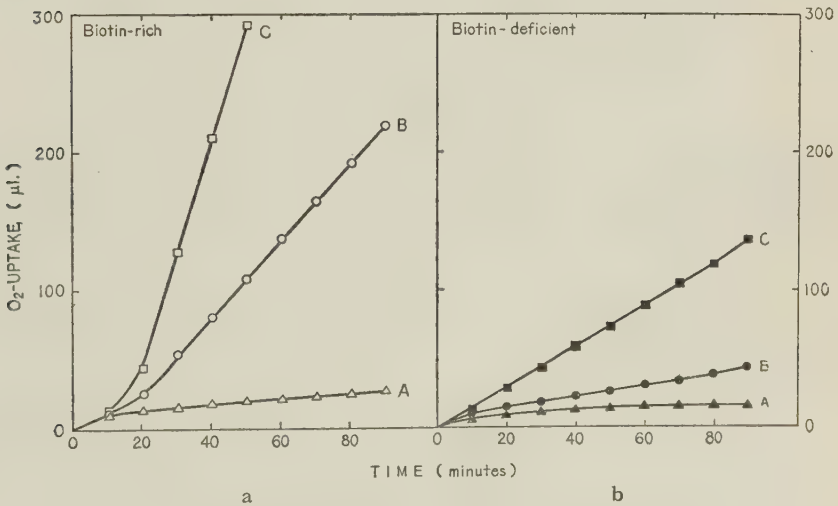

FIG. 1. Oxidation of pyruvate and glucose by resting cells.

The reaction was carried out in air at 30°. The main compartments contained cell suspension (biotin-rich cells, 13.1 mg. or biotin-deficient cells, 12.3 mg.) in 1/15 *M* phosphate buffer, pH 7.3, 1 μ mole of $MgSO_4$ and 1 μ mole of $MnSO_4$ in a final volume of 2.5 ml. The side arm contained 50 μ moles of glucose or sodium pyruvate in a volume of 0.5 ml. This was tipped in after equilibration. The center well contained 0.2 ml. of 40 per cent KOH. A: endogeneous, B: sodium pyruvate, C: glucose.

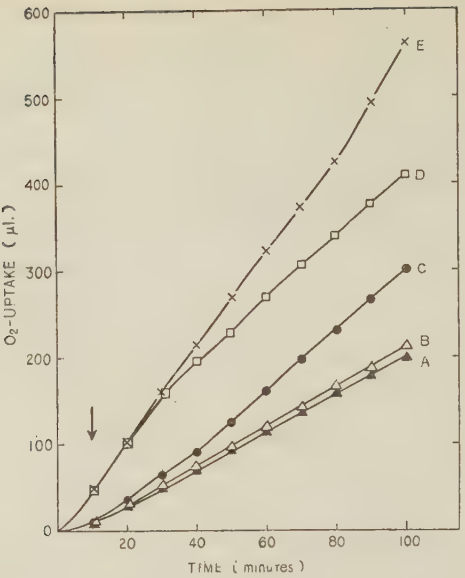

FIG. 2. Activation of the pyruvate oxidation of the biotin-deficient cells by biotin.

The biotin-deficient cell suspension (cells, 12.5 mg.) was preincubated with 500 mμg. of biotin (Curve C) or with 500 m μg of biotin and 50 μ moles of glucose (Curve D and E) for 3 hours at 37°. When most amount of glucose had been consumed, 50 μ moles of sodium pyruvate was added (Curve B, C and E) (shown by arrow). Other experimental conditions were the same as in Exp. 3 (Fig. 1) except that temperature was 37°.

The retardation of the pyruvate oxidation in the biotin-deficient cells was remarkably restored by the addition of DPN or ATP. On the other hand the stimulation by DPN or ATP was very slight in the biotin-rich cells as shown in Fig. 3.

The extent of the stimulation by the sufficient amount of DPN was almost the same as that caused by ATP. Furthermore, the stimulating effect of ATP after the addition of DPN was not observed and *vice versa* in the biotin-deficient cells.

From these results, it was concluded that the retardation of the pyruvate oxidation was not due to the decrease of the apoenzymes of pyruvic acid dehydrogenase system. And the stimulating effect of ATP was thought to consist in stimulating the DPN formation.

Dehydrogenation of Malate by Resting Cells (Exp. 6)—If the retardation of the pyruvate oxidation by the biotin-deficient cells is due to the decrease of DPN or ATP in the cells, the stimulating effects by these coenzymes should be observed in other dehydrogenase system which require DPN as coenzyme. Actually this was the case for example in the malate dehydrogenation system as shown in Table II.

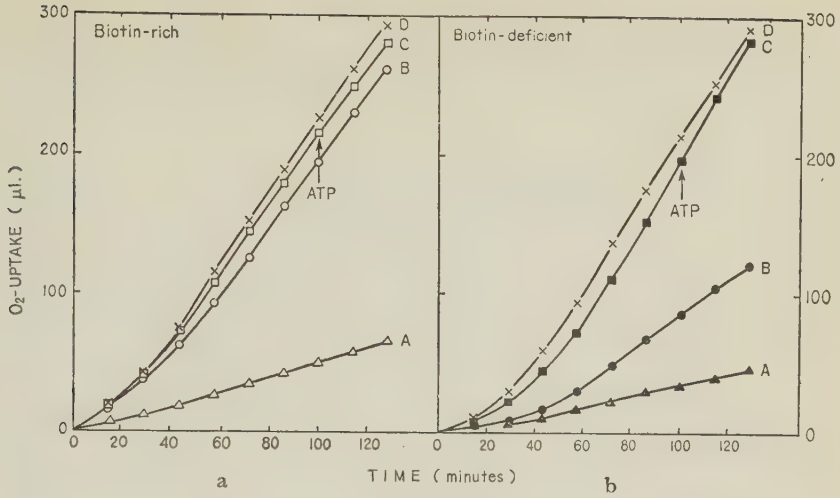

FIG. 3. Effect of DPN and ATP on the pyruvate oxidation by resting cells.

To the cell suspension (biotin-rich cells, 13.0 mg. or biotin-deficient cells 12.9 mg) 5 μM of DPN (Curve C) or 5 μM of ATP (Curve D) were added and preincubated for 30 minutes at 37°. Pyruvate was added from the first side arm (Curve C) and the reaction was proceeded at 37°. After 100 minutes ATP was added (shown by arrow). Curve A: endogeneous. Curve B: only pyruvate was added. Other experimental conditions were the same as in Exp. 3 (Fig. 1).

TABLE II

Effect of DPN and ATP on the Malate Dehydrogenation by the Resting Cells (Exp. 6)

The reaction mixture contained 0.5 ml. of 0.01 per cent methylene blue, 0.5 ml. of cell suspension (biotin-rich cells, 3.4 mg. or biotin-deficient cells, 3.2 mg.) in 1/15 *M* phosphate buffer, pH 7.3. To this mixture were added 1.0 ml. of 0.02 *M* sodium malate or 5 μ moles of DPN or 5 μ moles of ATP in the same buffer as indicated in the legends, Final volume, 3.0 ml. Temperature, 37°.

Conditions	Decoloration time (*min.*)
Biotin-rich cell	25
,, ,, +Malate	7 3/4
Biotin-deficient cell	20
,, ,, +ATP	18 1/5
,, ,, +DPN	19
,, ,, +Malate	15
,, ,, + ,, +DPN	8 1/3
,, ,, + ,, +ATP	8 1/10

The activity decreased in the biotin-deficient cells and the stimulating effects of DPN and ATP on the activity were observed as in the case of the

pyruvate oxidation.

Contents of Coenzymes in the Cells (Exp. 7)—The contents of ATP, DPN and CoA in the cells were determined with the lyophilized cells and the data are shown in Table III.

TABLE III

Contents of Coenzymes in the Cells (Exp. 7)

Cell	Coenzyme per 1 g. of cells ($\mu g.$)		
	ATP	DPN	CoA
Biotin rich cell	2700	2050	500
Biotin deficient cell	1200*	770	250

* This indicates that actual value were smaller than this because a large amount of inorganic phosphate interfered this assay value.

TABLE IV

DPN- and ATP- Decomposing Activity of the Resting Cells (Exp. 8, 9)

Exp. 8: To the 0.5 ml. of resting cell suspension (biotin-rich cells, 3.72 mg. or biotin-deficient cells, 4.14 mg.) in 1/15 *M* phosphate buffer, pH 7.3. or 0.5 ml. of cultural fluid, 0.45 μ mole of DPN was added and incubated at 37°. And then the reaction was stopped by the addition of 4 ml. of 1 *M* KCN and reaction mixture was centrifuged. The amount of DPN in the supernatant was determined by the cyanid addition method (*15*).

Exp. 9: The assay system contained 8.3 μ moles of ATP, 5 μ moles of $MgSO_4$ and 0.5 ml. of cell suspension (biotin-rich cells, 7.15 mg. or biotin-deficient cells, 7.06 mg.) in 0.06 *M* Tris buffer, pH 8.0, in a final volume of 1.0 ml. and the reaction mixture was incubated at 37°. The reaction was stopped by the addition of 2 ml. of 2.5 per cent TCA. The amount of inorganic phosphate was determined by the vanado-molybdate method (*17*). The decrease of ATP was calculated from the inorganic phosphate liberated.

	Time (*min.*)	Decrease of DPN/1 mg. of cell (μ *mole*)		
			Biotin-rich	Biotin-deficient
DPN-decomposing activity* (Exp. 8)	60	cell	0.0098	0.0148
		fluid	0.208	0.414
		total	0.218	0.429
	120	cell	0.0196	0.0262
		fluid	0.416	0.825
		total	0.435	0.856
	Time (*min.*)	Decrease of ATP/1 mg. of cell (μ *mole*)		
			Biotin-rich	Biotin-deficient
ATP-decomposing activity (Exp. 9)	120	cell	0.755	0.162

As in the case of *P. oryzae* (*7*), it seems that the contents of these coenzymes in the biotin-deficient cells have a tendency to decrease.

Decomposition of DPN and ATP by the cells (*Exp. 8, 9*)—To elucidate the mechanism of the decreasing phenomena of these coenzymes in the biotin-deficient cells, the DPN- and ATP-decomposing activities by these cells were investigated. As shown in Table IV the total DPN-decomposing activity (perhaps due to DPN-ase) of the biotin-deficient cells was found to be about twice as high as that of the biotin-rich ones as in the case of *Neurospora*. The larger part of the activity, however, was found to exist in the cultural medium and the smaller in the cells. On the other hand, the ATP-decomposing activity of the biotin-deficient cells showed remarkably lower value compared with that of the biotin-rich one.

Accordingly it was concluded that the decrease of ATP in the biotin-deficient cells was due to the depression of the ATP-producing activity in the cells.

Effect of AMP on the Pyruvate Oxidation by the Resting Cells (*Exp. 10*)—Under the assumption that the ATP formation was inhibited in the biotin-deficient

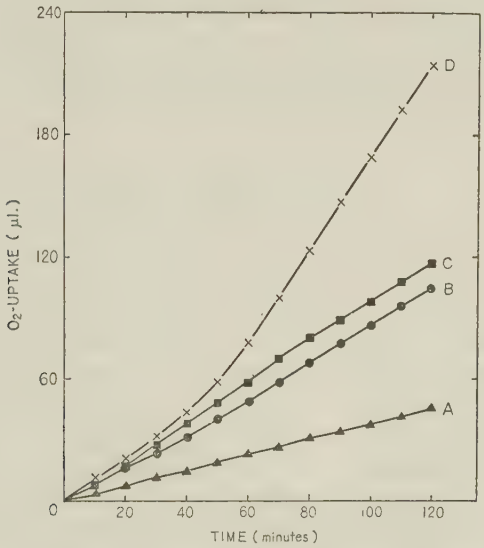

FIG. 4. Effect of AMP on the pyruvate oxidation by the biotin-deficient resting cells.

In this experiment each 10 μ moles of ATP or AMP were added to the biotin-deficient cell suspension (cells, 12.5 mg.) without preincubation. Other experimental conditions were the same as in Exp. 3 (Fig. 1). A: endogeneous, B: sodium pyruvate, C: AMP+sodium pyruvate, D: ATP+sodium pyruvate.

cells, the effect of AMP on the pyruvate oxidation was investigated to determine the point at which the formation of ATP was blocked by biotin-deficiency. The results obtained are presented in Fig. 4.

With addition of AMP only slight stimulation on the pyruvate oxidation was observed to occur contrary to the remarkable effect of ATP. These results suggest that the blockade produced by biotin-deficiency should exist in the process of phosphorylation of AMP.

Oxidative Phosphorylation by the Extracts of Cells (*Exp. 11*)—The cells, washed three times with 0.09 per cent sodium chloride at 0°, were suspended in 0.25 *M* sucrose solution and treated with sonic disintegrator for 5 minutes and centrifuged at 29,000×g for 30 minutes at 0°. The supernatant thus obtained was used as the extract of the cells for this experiment. The precipitated fraction, from which cell debris were previously removed by the centrifugation of 5,000× g for 30 minutes, scarcely showed the activity of the oxidative phosphorylation. This extract coupled the phosphorylation to the oxidation of exogeneous DPNH, the disapperance of which was used in this experiment as a measure of the

Table V

Oxidative Phosphorylation in the Extracts of the Cells. (*Exp. 11*)

Each cuvette contained in a final volume of 3 ml. the following: 100 μM of Tris buffer, pH 7.3, 1 μ mole of inorganic phosphate, 15 μ moles of magnesium chloride, 25 μ moles of sodium fluoride, 0.25 μ mole of ADP, 25 μ moles of glucose, 0.76 μ mole of DPNH and 0.1 ml. of the bacterial extract. When DNP was applied, 10^{-4} *M* DNP was added to this mixture. The reaction was started by the addition of the bacterial extract at 30°. The decrease in the absorbance at 340 mμ was measured after 4 minutes. The reaction was stopped by the addition of 1 ml. of cold 32 per cent TCA and the reaction mixture was centrifuged. The inorganic phosphate was determined with this supernatant.

Conditions	Dry weight of original cells (*mg.*)	ΔDPNH (*μ moles*)	ΔP (*μ moles*)	P : O
Biotin-rich extract	1.31	0.304	0.13	0.4
,, ,,		0.310	0.16	0.5
,, ,, +DNP		0.243	+	0
+DNP		0.233	+	0
Biotin-deficient extract	0.44*	0.330	0.064	0.19
,, ,,		0.334**	0.065	0.19
,, ,, +DNP		0.273	0	0
,, ,, +DNP		0.280	0	0

* Measurements were carried out after the original extract had been diluted three times because of strong activity of the DPNH oxidation.

** In other two series experiments, the DPNH oxidation was enhanced 300 to 800 per cent in the biotin-deficient extracts.

oxidation. Phosphorylation was measured by the diminution of inorganic phosphate added in the extract. ATP produced by the phosphorylation was fixed in glucose-6-phosphate by the hexokinase system which was added in the assay mixtures. The activity of the oxydative phosphorylation was compared between the extracts of the biotin-deficient cells and of the biotin-rich ones as shown in Table V.

Contrary to what was expected, the activity of the DPNH oxidation in

the biotin-deficient extract showed a strong increase in comparison with that of the biotin-rich one, while no remarkable difference was found in the activity of phosphorylation between both of the extracts. The phosphorylation was inhibited completely by the addition of 10^{-4} *M* DNP. But if ATP-decomposing activity shown in Table IV was proportional to the activity of ATPase and the correction of the ATP decomposition by ATPase was made on these values, the activity of the phosphorylation of the biotin-deficient cells would be higher. Accordingly the P : O ratios in the biotin-deficient extract showed remarkably lower value than those in the biotin-rich extract.

DISCUSSION

In 1942, Pilgrim and his collaborators (*1*) reported that the activity of the pyruvate oxidation by the liver homogenates of the biotin-deficient rat was depressed, but they could not find the stimulating effect of DPN contrary to the author's results (Exp. 5). The phenomena of the retardation of keto acid oxidation would be such a complicated one as shown in the author's results. This contradiction could not be explained exactly but would be perhaps caused by amounts of coenzymes concerned with this reaction, activities of enzymes which decomposed these coenzymes, permeability of coenzymes to mitochondria and other numerous factors in the cells.

At least in the author's experiments the retardation of the pyruvate oxidation in the biotin-deficient cells of *B. macerans* was found to be due to DPN-deficiency, and therefore, to ATP-deficiency. The relatively higher activity of glucose oxidation compared with pyruvate would be attributed to the formation of ATP at substrate oxidation level. From the results of Exp. 8 it was suggested that the decrease of DPN in the biotin-deficient cells might be due to the high DPNase activity. But the DPNase was found to be excreted into the culture medium during the cultivation and how much DPN was decomposed in the cell is rather doubtful. So it is possible to think that the decrease of DPN was not only due to the high DPNase activity but also due to the decrease of the DPN formation, though the proof for depression of the DPN-producing activity has not yet been obtained. In the author's opinion, the cause of depression of the DPN-producing activity in the biotin-deficient cells, for the most part, should not be due to the decrease of the DPN-synthesizing enzymes but to that of the ATP formation.

In 1954, Moat and his coworkers (*22*) found that 5-aminoimidazole ribotide, a precursor of ATP, was accumulated in the biotin-deficient yeast. Therefore there are at least two possible points in the biosynthetic pathway of ATP is blocked by biotin-deficiency. The first is the carbamylphosphorylation of 5-aminoimidazole ribotide. This seems probable since the retardation of carbamylphosphorylation of ornithine has already been noted in the biotin-deficient organism (*23*). The second is the phosphorylation of AMP by the oxidative phosphorylation. According to the author's experiment (Exp. 10) on the effect of AMP on the pyruvate oxidation, the latter eventuality seems probable, but the former possibility must not be excluded.

The results of the experiment on the oxidative phosphorylation indicate that the efficiency of the oxidative phosphorylation in the biotin-deficient cells decreases, in spite of the increase of the DPNH-oxidation activity. In connection with this, it is interesting that the activity of the ATP decomposition (perhaps of the ATPase) of the biotin-deficient cells decreases remarkably compared with the increase of ATPase under the effect of an uncoupler such as DNP. But as in the author's experiment the activity of the ATP decomposition was determined with the resting cells instead of the cell-free extract, further investigation about this problem should be waited for. The details of the experiment of the oxidative phosphorylation using the purified particulates of *B. macerans* will be reported in near future.

CONCLUSION

1. Under the biotin-deficient condition, *B. macerans* accumulated a great amount of pyruvate in the culture medium. While the resting cells of this bacteria accumulated α-ketoglutarate and pyruvate under aerobic condition.

2. Compared with the biotin-rich resting cells, marked depression was observed in the activity of keto acid oxidation by the biotin-deficient resting cells.

3. The retardation of the pyruvate oxidation by the biotin-deficient cells could be activated by preincubating the cells with biotin or better with biotin and glucose.

4. This retardation of the pyruvate oxidation could be restored by the addition of DPN or ATP. DPN was effective in the same degree as ATP or ATP and DPN.

5. Depressed activity of the malate dehydrogenation in the biotin-deficient resting cells was also restored by the addition of DPN or ATP.

6. Compared to the biotin-rich cells, the contents of the coenzymes such as ATP, DPN and CoA in the biotin-deficient cells was observed to decrease.

7. The DPN-decomposing activity of the biotin-deficient cells was remarkably higher than that of the biotin-rich cells. On the other hand the ATP-decomposing activity was proved to be very low.

8. AMP was effectless in the accerelation of the pyruvate oxidation by the biotin-deficient cells.

9. In the oxidative phosphorylation by the sonic disintegrated extract of the biotin-deficient cells, the activity of DPNH oxidation was found to increase remarkably compared with that of the biotin-rich cells, with no difference in the activity of the phosphorylation. Accordingly the P:O ratios showed remarkably lower values in the biotin-deficient extract.

The author wishes to express gratitude to Prof. S. Tanaka, Kyoto University, for many valuable criticisms and continued encouragement. And he is also indebted to Dr. T. Hasegawa, Institute for Fermentation, for the courtesy of furnishing *B. macerans*, and to Mr. J. Nagai, Mrs. F. Katsuki and Miss T. Yoshida for technical assistance in part.

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The Journal of Biochemistry, Vol. 46, No. 8, 1959

ACTION OF CHYMOTRYPSIN ON SYNTHETIC SUBSTRATES

III. ACTION OF α-CHYMOTRYPSIN ON AMINOACYL-L-TYROSINE AMIDES AND ETHYL ESTERS

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(Received for publication, November 24, 1958)

Previous communications from this laboratory have dealt with the action of chymotrypsin on various aminoacyl-L-tyrosine amides and ethyl esters in which the aminoacyl substituents are aliphatic amino acid groups (*1*, *2*). The data have shown that alteration in the aminoacyl substituents of aminoacyl-L-tyrosine amides and esters influences the rates of hydrolysis of the amide substrates and disappearance of the ester substrates by the enzyme.

In the present study, aminoacyl-L-tyrosine amides and ethyl esters, in which the aminoacyl residues are sarcosyl, L-prolyl, L-phenylalanyl, ε-acetyl-L-lysyl, and others were prepared and subjected to the action of chymotrypsin.

EXPERIMENTAL

Enzyme and Methods

Crystalline α-chymotrypsin was the same material as that previously described (*1*). The incubation was carried out at 30.0° in the presence of 0.1 *M* phosphate buffer at pH 8.0. The hydrolytic rates of the amide substrates were followed by means of the Conway microdiffusion method (*1*, *3*). The rates of disappearance of the ester substrates were followed by a modification of the hydroxamic acid method of Hestrin (*2*, *4*). Proteolytic coefficients (*C*) were estimated from $C=K/E$, where $K=(1/\text{min.})\log[100/(100-\%\text{ hydrolysis})]$ and E is the protein concentration in mg. of protein N per ml. of test solution.

Synthesis of Peptide Derivatives

Sarcosyl-L-tyrosinamide (SarTyrAm), sarcosyl-L-tyrosine ethyl ester (SarTyrOEt), L-prolyl-L-tyrosinamide (ProTyrAm), L-prolyl-L-tyrosine ethyl ester (ProTyrOEt), L-lysyl-L-tyrosinamide (LysTyrAm), ε-benzoyl-L-lysyl-L-tyrosinamide (ε-BzLysTyrAm), ε-acetyl-L-lysyl-tyrosinamide (ε-AcLysTyrAm) and ε-acetyl-L-lysyl-L-tyrosine ethyl ester (ε-AcLys TyrOEt) were prepared as described previously (*5*).

Carbobenzoxy-γ-benzyl-α-L-glutamyl-L-tyrosinamide—To the mixed anhydride (*6*) of carbobenzoxy-γ-benzyl-L-glutamic acid (0.01 mole) (*7*) and isobutyl chlorocarbonate (0.01 mole) in the presence of triethylamine (0.01 mole) and dimethylformamide (15 ml.) was added a mixture of L-tyrosinamide hydrochloride (0.01 mole), triethylamine (0.01 mole), and dimethylformamide (15 ml.). The reaction mixture was left overnight, and the crystals appearing after the addition of water were collected, and washed with

4 per cent bicarbonate solution, 2 per cent HCl, and water. The substance was recrystallized from methanol-water. Yield, 67 per cent; m.p. 171°; $[\alpha]_D^{25}$ −4.1° (c 2, in DMF (dimethylformamide)).

$C_{29}H_{31}O_7N_3$ (533.6): Calcd. C 65.3, H 5.9, N 7.9
Found. C 64.9, H 6.0, N 7.6

α-L-*Glutamyl*-L-*tyrosinamide* (*GluTyrAm*)—The above amide (0.005 mole) dissolved in a mixture of methanol (40 ml.) and acetic acid (15 ml.) was treated with hydrogen in the presence of palladium black. The filtrate was evaporated *in vacuo* repeatedly, water being added. The remaining crystals were collected and recrystallized from hot water-ethanol. The substance was sparingly soluble in cold water, but fairly soluble in an aqueous buffer at pH 8.0. Yield, 63 per cent; m.p. 200°.

$C_{14}H_{19}O_5N_3$ (309.3): Calcd. C 54.4, H 6.2, N 13.6
Found. C 53.9, H 6.2, N 13.6

L-*Phenylalanyl*-L-*tyrosine Ethyl Ester Hydrochloride* (*PheTyrOEt HCl*)—Carbobenzoxyphenylalanyltyrosine ethyl ester (0.005 mole) (*8*) dissolved in 0.2 *N* methanolic HCl (27 ml.) was hydrogenated in the usual manner (*1*, *2*, *5*), and the desired compound was obtained. Yield, 78 per cent; $[\alpha]_D^{20}$ +13.4° (c 2, in water).

$C_{20}H_{25}O_4N_2Cl \cdot H_2O$ (410.9): Calcd. C 58.5, H 6.6, N 6.8
Found. C 58.9, H 6.8, N 6.7

L-*Phenylalanyl*-L-*tyrosinamide Hydrochloride* (*PheTyrAm HCl*)—The compound was obtained from carbobenzoxyphenylalanyltyrosinamide (*8*, *9*) as described above. Yield, 92 per cent; m.p. 233–235° (decomp.); $[\alpha]_D^{20}$ +25.3° (c 2, in water).

$C_{18}H_{22}O_3N_3Cl$ (363.8): Calcd. C 59.4, H 6.1, N 11.6
Found. C 59.1, H 6.2, N 11.4

The preparation of PheTyrAm was reported in the literature (*8*).

Carbobenzoxy-L-*tryptophyl*-L-*tyrosinamide*—The mixed anhydride of carbobenzoxy-L-tryptophan (*9*) in tetrahydrofuran was coupled with tyrosine ethyl ester in the usual manner (*1*, *6*). The oily carbobenzoxytryptophyltyrosine ethyl ester obtained was converted to the crystalline amide with an over-all yield of 58 per cent; m.p. 201–202°; $[\alpha]_D^{15}$ −46.8° (c 2, in DMF).

$C_{28}H_{28}O_5N_4$ (500.5): Calcd. N 11.1
Found. N 11.1

L-*Tryptophyl*-L-*tyrosine Ethyl Ester Hydrochloride* (*TryTyrOEt*)—The compound was obtained from the oily carbobenzoxy ester in non-crystalline powder. Yield, 81 per cent; $[\alpha]_D^{20}$ +27.6° (c 2, in water).

$C_{22}H_{26}O_4N_3Cl \cdot 1/2H_2O$ (440.9): Calcd. C 59.9, H 6.2, N 9.5
Found. C 60.0, H 6.6, N 9.4

L-*Tryptophyl*-L-*tyrosinamide Hydrochloride* (*TryTyrAm HCl*)—The compound was obtained from the crystalline carbobenzoxy amide. Yield, 76 per cent; $[\alpha]_D^{20}$ +55.9° (c 2, in water).

$C_{20}H_{23}O_3N_4Cl \cdot H_2O$ (420.9): Calcd. C 57.1, H 6.0, N 13.3
Found. C 57.7, H 6.3, N 12.7

Carbobenzoxy-D-*tryptophyl*-L-*tyrosinamide*—The coupling reaction of carbobenzoxy-D-tryptophan (*10*) and tyrosine ester gave an oily carbobenzoxy ester, which was converted to the crystalline amide with an over-all yield of 46 per cent; m.p. 199–200°; $[\alpha]_D^{15}$ +10.0° (c 2, in DMF).

$C_{28}H_{28}O_5N_4$ (500.5): Calcd. N 11.1
Found. N 11.0

D-*Tryptophyl*-L-*tyrosine Ethyl Ester Hydrochloride* (D-*TryTyrOEt HCl*)—The compound was obtained from the oily carbobenzoxy ester in non-crystalline powder with a yield of 84 per cent.

$C_{22}H_{26}O_4N_3Cl \cdot 1/2H_2O$ (440.9): Calcd. C 59.9, H 6.2, N 9.5
Found. C 59.4, H 6.6, N 9.4

D-*Tryptophyl*-L-*tyrosinamide Hydrochloride* (D-*TryTyrAm HCl*)—The compound was obtained from the carbobenzoxy amide in non-crystalline form. Yield, 85 per cent; $[\alpha]_D^{20}-22.3°$ (c 2, in water).

$C_{20}H_{23}O_3N_4Cl \cdot H_2O$ (420.9): Calcd. C 57.1, H 6.0, N 13.3
Found. C 57.5, H 6.4, N 12.9

α-Acetyl-ε-carbobenzoxy-L-*lysyl*-L-*tyrosinamide*—The coupling reaction of α-acetyl-ε-carbobenzoxylysine (*11*) and tyrosine ester gave an oily carbobenzoxy ester, which was converted to the amide with an over-all yield of 72 per cent; m.p. 171°; $[\alpha]_D^{16}-15.0°$ (c 2, in DMF).

$C_{25}H_{32}O_6N_4$ (484.5): Calcd. N 11.6
Found. N 11.5

α-Acetyl-L-*lysyl*-L-*tyrosinamide Hydrochloride* (*α-AcLysTyrAm HCl*)—The compound was obtained from the above amide in non-crystalline powder. Yield, 94 per cent; $[\alpha]_D^{20}-5.7°$ (c 2, in water).

$C_{17}H_{27}O_4N_4Cl$ (386.9): Calcd. C 52.8, H 7.0, N 14.5
Found. C 52.4, H 7.5, N 13.7

RESULTS

Proteolytic Coefficients and Reaction Kinetics of the Amide Substrates—The compounds were tested at $0.05 \sim 0.0025\ M$ initial substrate concentrations. The determinations for two substrates, ε-AcLysTyrAm and ε-BzLysTyrAm, were made in 30 volumes per cent methanol because of their insolubility in an aqueous buffer. For comparison, the determination for GlyTyrAm in aqueous methanol was carried out.

Representative data for the hydrolysis of ProTyrAm, PheTyrAm and ε-AcLysTyrAm are shown in Table I as an example. It was observed that the hydrolysis of some substrates such as SarTyrAm or ProTyrAm followed first order kinetics. However, in the hydrolysis of other substrates such as PheTyrAm, the values of proteolytic coefficients decreased progressively. In this case, an extraporated initial value was taken for comparison of sensibility of a substrate (*1*, *12*). A summary of all coefficients determined are given in Table II.

Measurements of initial rates of hydrolysis in different concentrations of SarTyrAm, ProTyrAm, PheTyrAm, TryTyrAm and LysTyrAm gave the values of K_m and k_3 at pH 8.0 and 30° by the use of the plotting method of Lineweaver and Burk (*1*). Plots for the hydrolysis of the substrates are shown in Fig. 1. The values of K_m, k_3 and $C_{max}=k_3/(2.3\,K_m)$ obtained are shown in Table III.

Table I

Example in Measurements of Hydrolysis of Aminoacyl-L-tyrosinamides by Chymotrypsin

Substrate concentration, 0.01 *M*; pH 8.0 (0.1 *M* phosphate buffer); temperature, 30°.

Substrate	Enzyme concentration (*mg. Protein N per ml.*)	Time (*min.*)	Hydrolysis (*per cent*)	Proteolytic coefficient
ProTyrAm	0.0265	20.0	13.9	0.123
		37.2	22.4	0.112
		54.5	33.4	0.122
		70	40.5	0.121
		84	48.0	0.127
Average				0.121
PheTyrAm[a)]	0.1005	20.5	16.0	0.0367
		40.0	27.5	0.0348
		55.0	35.0	0.0338
		75	39.2	0.0286
		91	43.3	0.0269
		102	43.0	0.0238
Extraporated value				0.041
ε-AcLysTyrAm[b)]	0.388	15.5	10.2	0.0078
		28.0	17.3	0.0076
		39.0	22.8	0.0074
		50.7	27.5	0.0071
		64	32.6	0.0069
		79	37.6	0.0067
Extraporated value				0.0081

a) Fruton and Bergmann determined per cent hydrolysis of this substrate after 22 hours incubation with chymotrypsin (*8*).

b) Measurements were made in 30 volumes per cent methanol.

Disappearance of the Ester Substrates in the Absence and the Presence of Enzyme It was determined that the non-enzymatic disappearance of the substrates tested followed first order kinetics within the limits of experimental error. The values of velocity constants ($K_{no\ enz.}$) for the substrates are given in Table V. It was observed that, although GlyTyrOEt (in 30 per cent methanol), SarTyrOEt and ProTyrOEt produced no precipitates during the incubation, precipitates of diketopiperazines of the other ester substrates appeared within 1~20 hours in the absence of the enzyme.

Representative data for the disappearance of ε-AcLysTyrOEt and PheTyr OEt in the presence of the enzyme are given in Table IV as an example.

Table II

Proteolytic Coefficients of Aminoacyl-L-tyrosinamides at Various Initial Substrate Concentrations

pH 8.0; temperature, 30°.

Substrate	Proteolytic coefficient, *C*				
	0.05 *M*	0.025 *M*	0.01 *M*	0.005 *M*	0.0025 *M*
GlyTyrAm[a]	0.017	0.020	0.021		
SarTyrAm		0.028	0.034	0.043	
ProTyrAm		0.095	0.121	0.166	
GluTyrAm	0.0103	0.0118	0.0120		
PheTyrAm			0.041[b]	0.078[b]	0.114[b]
TryTyrAm			0.0199[b]	0.032[b]	0.076[b]
D-TryTyrAm			0.0046[b]		
LysTyrAm	0.030	0.053	0.080		
α-AcLysTyrAm			0.061[b]		
ε-BzLysTyrAm[c]			0.05		
GlyTyrAm[d,e]			0.0035[b]		
ε-AcLysTyrAm[d]			0.0081[b]		
ε-BzLysTy.Am[d]			0.0053[b]		

a) The data are from Izumiya and Yamashita (*1*).

b) The values given are extraporated initial constants, since decreasing values of C were observed.

c) This was partly soluble in the aqueous buffer, therefore the suspension was applied for the enzymatic test.

d) Measurements were made in 30 per cent methanol.

e) Kaufman *et al.* reported that GlyTyrAm in 30 per cent methanol is hydrolyzed at 0.19 times the rate in aqueous solution (*13*).

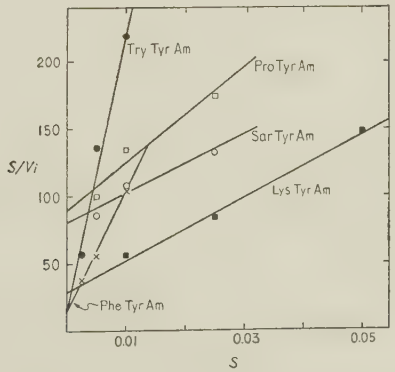

FIG. 1. The plots of initial substrate concentration [*S*] divided by initial velocity V_i *versus* [*S*] for the hydrolysis of the substrates at pH 8.0 and 30°. The enzyme concentrations were 0.1175 mg. (SarTyrAm), 0.0265 mg. (ProTyrAm), 0.1005 mg. (PheTyrAm and TryTyrAm), and 0.0976 mg. protein N per ml. (LysTyrAm).

Table III

Kinetic Constants of Aminoacyl-L-tyrosinamides

pH 8.0; temperature, 30°.

Substrate	K_m (M)	k_3[a]	C_{max}
GlyTyrAm[b]	0.15	0.0078	0.023
SarTyrAm	0.039	0.0041	0.046
ProTyrAm	0.026	0.011	0.18
PheTyrAm	0.0015	0.0011	0.32
TryTyrAm	0.00075	0.00048	0.28
LysTyrAm	0.013	0.0044	0.15

a) In M/liter/min./mg. protein N/ml.
b) The data are from Izumiya and Yamashita (*1*).

Table IV

Example in Measurements of Disappearance of Aminoacyl-L-tyrosine Ethyl Esters in the Presence of Chymotrypsin

Substrate concentration, 0.01 M; pH 8.0; temperature, 30°.

Substrate	Enzyme concentration (10^{-3} *mg. protein N per ml.*)	Time (*min.*)	Disappearance (*per cent*)	$K_{obs.}$[a]
ε-AcLysTyrOEt[b]	0.242	6.5	24.8	0.0190
		10.1	34.2	0.0180
		14.0	44.7	0.0184
		18.3	53.0	0.0179
		22.5	62.0	0.0187
Average				0.0184
PheTyrOEt[b,c]	0.242	3.1	3.9	0.0056
		6.2	7.4	0.0054
		10.2	11.7	0.0053
		15.1	15.1	0.0047
		19.2	18.6	0.0047
Extraporated value				0.0057

a) K=(1/min.) log [100/(100−% ester disappearance)].
b) A gelatinous precipitate began to appear after 1∼3 minutes.
c) Measurements were made in 30 per cent methanol.

The values of velocity constants ($K_{obs.}$) for the substrates tested are given in Table V. It should be noted that, when progressive diminutions of the constants were found, extraporated initial values were used (*2*). The values of first order velocity constants by enzymatic action ($K_{enz.}$), which are shown in

Table V, are derived from $K_{obs.}-K_{no\ enz.}$. The apparent proteolytic coefficients ($C=K_{enz.}/E$) shown in Table V may be taken as approximate measures of the relative susceptibility of the ester substrates by the enzyme. It was observed that fairly massive precipitates appeared within 1~6 minutes in the case of PheTyrOEt, TryTyrOEt, ε-AcLysTyrOEt and D-TryTyrOEt, but no precipitates appeared in the cases of GlyTyrOEt (in aqueous methanol), SarTyrOEt and ProTyrOEt. Though the precipitates might be produced by transpeptidation reaction, their natures have not been identified yet.

TABLE V

Proteolytic Coefficients in Disappearance of Aminoacyl-L-tyrosine Ethyl Esters

Substrate concentration, 0.01 *M*; pH 8.0; temperature, 30°.

Substrate	$K_{no\ enz.}$	Enzyme concentration (10^{-3} *mg. protein N per ml.*)	$K_{obs.}$	$K_{enz.}$[a]	Proteolytic coefficient, C[a]
GlyTyrOEt[b]	0.0047	0.220	0.036	0.031	141
SarTyrOEt	0.0020	0.242	0.040	0.038	157
ProTyrOEt	0.0007	0.242	0.023	0.022	91
ε-AcLysTyrOEt	0.0012	0.242	0.0184	0.0172	71
GlyTyrOEt[c]	0.0046	0.242	0.0080	0.0034	14.1
PheTyrOEt[c]	0.0010	0.242	0.0057[d]	0.0047	19.4
TryTyrOEt[c]	0.0031	6.12	0.066[d]	0.063	10.3
D-TryTyrOEt[c]	0.0009	6.05	0.0093	0.0084	1.4

a) $K_{enz.}=K_{obs.}-K_{noe\ nz.}$. $C=K_{enz.}/E$.
b) The data are from Izumiya and Yamashita (*2*).
c) Measurements were made in 30 per cent methanol.
d) The values given are extraporated initial constants.

Paperchromatography of Reaction Mixture—In addition to measurements by Conway method of the amount of NH_3 liberated or by Hestrin method of the extent of disappearance of ester, the course of the enzymatic reaction was followed qualitatively by means of paperchromatography (*1*, *2*). The R_f values of the reference compounds are given in Table VI. In the cases of the amide substrates except PheTyrAm and TryTyrAm, hydrolysis of the substrates occurred, yielding free aminoacyltyrosines. The reaction mixture of the enzyme and PheTyrAm or TryTyrAm showed 2~3 spots of unknown substances on chromatograms. Although the nature of the unknown substances could not be identified, PheTyrAm and TryTyrAm might be hydrolyzed at the peptide bond as well as amide bond, as shown by Blau and Waley (*14*).

In the cases of the ester substrates in the presence of the enzyme, the faint spots of free dipeptides were revealed on the chromatogram; the results may be explained by the fact that the enzyme catalyzes the formation of free dipeptide and of ninhydrin negative products from the ester substrate as shown in the previous communication (*2*).

Table VI

R_f Values of Reference Compounds

The compounds were chromatographed on Toyo Roshi No. 50 paper using the solvent systems of *n*-butanol : acetic acid : pyridine : water (15 : 3 : 10 : 12, and 4 : 1 : 1 : 2, *v/v*). The ascending technique was applied.

Substance	R_f	
	(15 : 3 : 10 : 12)	(4 : 1 : 1 : 2)
SarTyrAm	0.55	0.43
SarTyrOEt	0.84	0.79
(SarTyr)	0.47[a]	
ProTyrAm	0.62	
ProTyrOEt[b]	—	—
ProTyr[c]	0.55	
GluTyrAm	0.46	0.37
GluTyr[c]	0.40	0.36
PheTyrOEt		0.90
(PheTyr)		0.72[a]
PheTyrAm		0.75
TryTyrOEt		0.90
TryTyrAm		0.70
LysTyrAm	0.39	0.28
LysTyr[c]	0.33	0.22
α-AcLysTyrAm	0.54	
(α-AcLysTyr)	0.46[a]	
ε-AcLysTyrAm	0.64	0.52
ε-AcLysTyrOEt		0.79
(ε-AcLysTyr)		0.50[a]
ε-BzLysTyrAm	0.82	0.74 (0.67[d])
(ε-BzLysTyr)	0.82[a]	0.74[a] (0.76[d])

a) A new spot of this R_f value arised in an enzymatic reaction with an amide or ester substrate. An unknown substance of this R_f was assumed to be the dipeptide which is shown in parentheses, since no synthetic peptide was available.

b) The sensibility for development by ninhydrin was so poor that the spot could not be revealed.

c) The synthesis has been described in the communication (*10*).

d) The solvent system of *tert*-butanol : formic acid : water (15 : 3 : 2, *v/v*) was applied.

DISCUSSION

The previous study in this laboratory has shown that the hydrolytic rates by chymotrypsin of many aminoacyl-tyrosinamides, in which the aminoacyl

substituents are the aliphatic amino acid groups, were influenced to some extent by alteration of the N-terminal amino acid residues of the substrates (*1*). The present results shown in Tables II and III demonstrate also that alteration of the N-terminal amino acid residues of the amide substrates influences the hydrolytic rates; ProTyrAm is hydrolyzed at the highest rate among the 10 kinds of the amide substrates tested. It would be noteworthy that the values of K_m obtained for PheTyrAm and TryTyrAm, in which L-phenylalanine and L-tryptophan are known as the sensitive amino acids for chymotrypsin, are significantly less than that of any of the others in Table III and the substrates shown in the communication (*1*); the results may be explained as owing to the faster rates of complex formation. Attention should be given, however, that the determination of kinetic constants for PheTyrAm and TryTyrAm are carried out on the basis of the assumption that hydrolysis at the amide bond is the sole enzymatic reaction at initial stage.

Clark-Lewis and Fruton reported that γ-L-Glu-L-PheAm, was hydrolyzed by chymotrypsin at 0.13 times the rate for GlyPheAm (*15*). In the present study, the ratio of 0.57 to 1 is obtained for α-GluTyrAm to GlyTyrAm (Table II). The result may be explained that the presence of acidic γ-carboxyl group in α-GluTyrAm is unfavorable for the hydrolysis by the enzyme, although more specific factors may also be involved in the interaction of the enzyme and each of the two substrates, α-GluTyrAm and GlyTyrAm.

Izumiya and Fruton reported that LysTyrAm was resistant entirely to the action of cathepsin C, although ε-AcLysTyrAm was hydrolyzed very slowly (*5*)*. In the present study, however, it is shown that LysTyrAm is rapidly attacked by chymotrypsin, and ε- or α-acyllysyl derivatives are also hydrolyzed by the enzyme at somewhat lesser extent than LysTyrAm (Table II).

In the previous communication, it has been shown that the disappearance rates of the ester substrates by chymotrypsin were influenced by alteration of the N-terminal amino acid residues of aminoacyltyrosine ethyl esters (*2*). In the present study, it also is shown that alteration of the N-terminal amino acid residue influences the disappearance rates of the ester substrates as listed in Table V. It should be noted, however, that the validity of the comparison of the susceptibility is limited because the determinations were made at a single initial substrate concentration. This problem has been discussed by Neurath and Schwert (*16*).

SUMMARY

1. A number of aminoacyl-L-tyrosinamides in which the aminoacyl residues are sarcosyl, L-prolyl, α-L-glutamyl, L-phenylalanyl, L- and D-tryptophyl, L-lysyl, α- and ε-acetyl-L-lysyl, and ε-benzoyl-L-lysyl have been tested as substrates for α-chymotrypsin at pH 8.0° and 30°.

* During the stay of the present author (N. I.) in Fruton's laboratory of Yale University, Prof. Joseph S. Fruton provided the helpful suggestion that chymotrypsin might hydrolyse LysTyrAm.

2. The values of proteolytic coefficients in various initial substrate concentrations of the amide substrates were determined. The values of K_m, k_3 and C_{max} of some amide substrates were obtained. By the comparison of the proteolytic coefficients, it was found that the hydrolytic rates of the substrates were influenced to some extent by alteration of the N-terminal amino acid residues of the aminoacyltyrosinamides.

3. The disappearance rates of 0.01 *M* aminoacyl-L-tyrosine ethyl esters in which the aminoacyl residues are sarcosyl, L-prolyl, L-phenylalanyl, L- and D-tryptophyl, and ε-acetyl-L-lysyl were determined in the absence and presence of the enzyme.

4. The courses of the enzymatic reactions were analyzed by means of paperchromatography.

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The Journal of Biochemistry, Vol. 46, No. 8, 1959

DENATURATION AND INACTIVATION OF ENZYME PROTEINS

XII. THERMAL INACTIVATION AND DENATURATION OF GLUTAMIC ACID DEHYDROGENASE AND THE EFFECT OF ITS COENZYME ON THESE PROCESSES*

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(Received for publication, November 27, 1958)

Kubo (*1*) has reported that glutamic acid dehydrogenase was protected from thermal inactivation by its coenzyme. However it was reported in the preceding paper (*2*) that the reduced form of diphosphopyridine nucleotide, unlike the oxidized form, did not protect glutamic acid dehydrogenase from inactivation and denaturation by urea, but instead greatly accelerated these processes. In an attempt to clarify the effect of the reduced coenzyme on the inactivation and denaturation of the enzyme protein, an examination was made of the effect of the DPN-CN complex on thermal inactivation of glutamic acid dehydrogenase, because it has been shown that diphosphopyridine nucleotide reacts with cyanide to form a DPN-CN complex having an analogous structure to the reduced form of the nucleotide (*3*).

Protein has been shown to be reduced by incubation for long period with a high concentration of cysteine (*4*). Negelein *et al.* (*5*) suggested that the coenzyme might be reduced by hydrogen transferred from the sulfhydryl group in yeast alcohol dehydrogenase, because the coenzyme was reduced after incubation with the enzyme in the absence of substrate. Therefore in the present investigation a study was made of whether there is a relation between the effect of glutamic acid dehydrogenase on the coenzyme in the absence of substrate and the effect of cysteine on the stability of the enzyme.

The present paper deals with the effects of the coenzymes, the DPN-CN complex, and cysteine on the stability of glutamic acid dehydrogenase.

EXPERIMENTAL

Crystalline glutamic acid dehydrogenase of pig liver, bacterial proteinase, and

* An outline of this work was presented at the 10th Meeting of the Symposia on Enzyme Chemistry, Japan, at Sapporo, in July, 1958.

The following abbreviations are used in the present paper: GADH, glutamic acid dehydrogenase; DPN, DPN^+, and DPNH, diphosphopyridine nucleotide, oxidized form, and reduced form, respectively.

the oxidized and reduced forms of diphosphopyridine nucleotide were prepared according to the methods described in the preceding paper (*2*).

The activity of glutamic acid dehydrogenase was determined spectrophotometrically by measuring the oxidation rate of DPNH. The assay system was the same as described in the preceding paper (*2*). The enzyme activity is represented here by $10^3 \times \Delta E_{340}$ per minute where ΔE_{340} is the decrease in optical density of the assay mixture at 340 mμ after the addition of the enzyme solution.

The "ratio of inactivation" and "per cent residual activity" were calculated from the equation given in the preceding paper (*2*).

The "ratio of denaturation" was determined by the bacterial proteinase method as described in the preceding paper (*2*).

RESULTS

Effect of Coenzyme on Thermal Inactivation and Denaturation of Glutamic Acid Dehydrogenase—It has been shown that glutamic acid dehydrogenase is protected from thermal inactivation by the presence of a high concentration of sodium sulfate (*6*). However, in the presence of a low concentration of the salt, sixty

Table I

Effect of Coenzyme and Substrate on Thermal Inactivation of Glutamic Acid Dehydrogenase

Concentrations: GADH, 0.002 per cent; DPN, 82 μM; glutamate, 0.133 *M*. Total volume of reaction mixture, 3.0 ml. 0.1 ml. of reaction mixtures was used to test the activity.

Additions[1)]	Heat-traetment	Activity	Per cent residual activity
None	—	79	100
None	55°, 3 mins.	57	72
DPN^+	55°, 3 mins.	42	53
DPNH	55°, 3 mins.	5	6.3
None	—	85	100
None	55°, 5 mins.	30	35.3
DPN^+	55°, 5 mins.	47	55.0
DPNH	55°, 5 mins.	0	0.0
None	—	83	100
None	55°, 5 mins.	30	37.3
DPN^+	55°, 5 mins.	36	43.4
DPNH	55°, 5 mins.	6	7.2
Glutamate	55°, 5 mins.	54	65.1
DPN^+ + glutamate	55°, 5 mins.	5	6.2

1) Additions were preincubated with GADH at 30° for 30 minutes prior to heat-treatment.

per cent of the original activity of the dehydrogenase was lost by heating at 55° for five minutes as shown in Table I. Thermal inactivation as well as urea inactivation (*2*) were depressed by the addition of DPN^+ or glutamate, but were increased by the addition of DPNH or a mixture of DPN^+ and glutamate as shown in Table I.

A relationship between the stimulatory effect and the concentration of DPNH was examined. When 0.1 μM of enzyme was heated at 50° for ten minutes, it was completely inactivated in the presence of 50 μM of DPNH but unaffected in the absence of DPNH (Table II).

TABLE II

Effect of Various Concentrations of DPNH on Thermal Inactivation of GADH

Conc. of DPNH[1], (μM)	Heat-treatment[2]	Activity[3]	Per cent residual activity
0	−	68	100
	+	68	100
7.5	−	81	100
	+	48	60.7
10.0	−	82	100
	+	31	38.2
12.5	−	84	100
	+	12	14.6
30.0	−	99	100
	+	6	7.2
50.0	−	105	100
	+	0	0.0
75.0	−	118	100
	+	0	0.0

1) GADH, 0.01 per cent. Preincubated with GADH at 30° for 30 minutes prior to heat-treatment.

2) 50° for 10 minutes.

3) After cooling, 0.1 ml. of heated mixture was added to the assay system and the activity was measured immediately.

The effect of DPNH on thermal denaturation of this enzyme was also examined. Table III shows that DPNH accelerates not only thermal inactivation but also thermal denaturation of glutamic acid dehydrogenase.

Effect of Analogues of DPNH on Thermal Inactivation of Glutamic Acid Dehydrogenase—It has been reported that DPN^+ reacts in alkaline solution with cyanide to form a complex, in which the cyanide occupies the para-position of the pyridine ring (*3*). Hydrogen transport by pyridine nucleotide has been shown to take place through the para-position of the nicotine amide moiety (*7*). Therefore, the DPN-CN complex was used as an analogue of DPNH. After preincubation of glutamic acid dehydrogenase with DPN^+, cyanide, or a mixture of DPN^+ and cyanide, the enzyme was heated at 52° for ten minutes. Table IV shows that the thermal inactivation of the enzyme was accelerated by the addition of a mixture of DPN^+ and cyanide, whereas no acceleration was observed when either was added alone. The DPN-CN complex is formed

Table III

Effect of Coenzymes on Thermal Denaturation and Inactivation of Glutamic Acid Dehydrogenase

Reaction mixture: GADH, 0.04 per cent; DPN^+, 0.35 mM; DPNH, 0.35 mM; total volume, 2.5 ml.; pH, 7.4. Heat-treatment was carried at 50° for 10 minutes. After heating and then cooling immediately in ice-cold water, 2.0 ml. of reaction mixture was incubated with 0.3 ml. of 0.15 per cent solution of bacterial proteinase at 30° for 20 minutes. The proteolytic reaction was stopped by addition of 0.3 ml. of 4 *M* trichloroacetic acid. The resulting suspension was filtered through filter paper. 1.0 ml. of the filtrate was used for the Folin-Ciocalteu reaction and the optical density was measured.

After cooling, 0.2 ml. of heated reaction mixture was diluted with 3.8 ml. of 0.1 *M* phosphate buffer, pH 7.4. 0.2 ml. of the diluted solution was used to measure the activity.

Additions	Heat-treatment	Activity	Ratio of inactivation	Folin color	Ratio of denaturation
None	−	66	—	0	—
None	+	73	0	0	0
None	+[1]	—	—	0.226	100
DPN^+	−	75	—	0	—
DPN^+	+	69	7.9	0.003	1.3
DPNH	−	77	—	0	—
DPNH	+	48	37.7	0.046	20.4

1) Completely denatured at 90° for 10 minutes.

Table IV

Effect of DPN-CN Complex on Thermal Inactivation of GADH

Addition[1]		Heat-treatment[2]	Activity[3]	Per cent residual activity
DPN (μM)	KCN (mM)			
—	—	−	82	100
—	—	+	36	43.9
100	—	+	40	48.8
—	5	+	45	54.8
100	5	+	16	19.5
—	15	+	45	54.8
100	15	+	10	12.2
—	28	+	45	54.8
100	28	+	4	4.9

1) Additions were preincubated with GADH, 0.002 per cent, at 30° for 30 minutes prior to heat-treatment. pH 8.0.

2) 52° for 10 minutes.

3) 0.1 ml. of heated mixture was used to measure activity.

well at a strongly alkaline pH, but only slightly at pH 8.0. It seems likely that the acceleration of thermal inactivation of the dehydrogenase was caused by a very small amount of DPN-CN complex present.

It has been shown that hydroxylamine combines in the para-position with the nicotine amide moiety of DPN only in the presence of alcohol dehydrogenase (*8*). If this is so, it is to be expected that the mixture of DPN^+ and hydroxylamine, as an analogue of DPNH, is unsuitable for the experiments with glutamic acid dehydrogenase mentioned above. As expected the mixture did not accelerate the thermal inactivation of glutamic acid dehydrogenase, but markedly protected the enzyme from inactivation. As shown in Table V, the addition of hydroxylamine alone decreased the thermal inactivation of the enzyme. Therefore it seems probable that the DPN-NH_2OH complex is not formed in the presence of glutamic acid dehydrogenase and the protective action observed by the addition of the mixture is due, not to the complex formed, but to the action of hydroxylamine alone. Hence it follows that hydroxylamine is capable of acting on glutamic acid dehydrogenase as

Table V

Effect of Hydroxylamine on Thermal Inactivation of GADH

Additions[1]	Heat-treatment[2]	Activity[3]	Per cent residual activity
None	−	47	100
None	+	12	25.6
0.28 *M* NH_2OH	−	48	100
0.28 *M* NH_2OH	+	41	85.5
0.28 *M* NH_4Cl	−	54	100
0.28 *M* NH_4Cl	+	47	92.5
0.37 *M* glutamate	−	47	100
0.37 *M* glutamate	+	37	78.9
0.28 *M* urea	−	49	100
0.28 *M* urea	+	11	22.2

1) Additions were preincubated with GADH, 0.001 per cent, at 30° for 30 minutes prior to heat-treatment. pH 8.0.

2) 53° for 10 minutes.

3) 0.1 ml. of heated mixture was used to measure activity.

an analogue of substrate having a protective action, such as the ammonium ion and glutamate.

Oxidation of Reduced Coenzyme by Glutamic Acid Dehydrogenase without Substrate—When a high concentration of glutamic acid dehydrogenase was mixed with DPNH in the absence of substrate, the optical density at 340 mμ decreased slowly. The spectrum after incubation for 24 hours has a peak at 340 mμ like that of DPNH alone, as shown in Fig. 1.

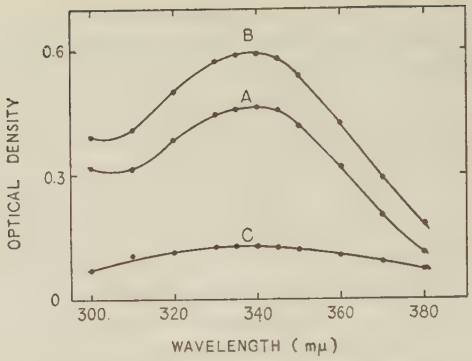

FIG. 1. Spectrum of DPNH after 24 hours incubation with GADH.

Curve A: Spectrum of DPNH after 24 hours incubation with GADH. Curve B: Spectrum calculated from control spectrums of GADH and DPNH after 24 hours incubation. Curve C: Difference spectrum between A and B.

TABLE VI

Oxidation of DPNH by GADH without Substrate

GADH (*μg./ml.*)	$10^3 \times \Delta E_{340}$ Incubation time		Oxidized DPNH for 40 hours (*μM*)
	15 hrs.	40 hrs.	
0.191	33	30	4.3
0.373	63	65	10.5
0.758	115	165	26.6

The maximum decrease in optical density at 340 m*μ* was nearly proportional to the concentration of enzyme, as shown in Table VI.

However, when glutamic acid was added into the incubation mixture containing enzyme and DPNH the optical density at 340 m*μ* increased very rapidly. It seems that reduced coenzyme was oxidized by the enzyme to DPN^+ which was again reduced by the addition of glutamic acid.

Effect of Cysteine on Thermal Inactivation of Glutamic Acid Dehydrogenase— When glutamic acid dehydrogenase was incubated with cysteine prior to heat-treatment, thermal inactivation of the enzyme was almost completely prevented regardless of the length of the preincubation period as shown in Table VII. The protective action of cysteine was compared with the action of other amino acids with similar structures. As shown in Table VIII, other amino acids such as serine, alanine, and aspartate, had no protective action. It is, therefore, conceivable that the protective action of cysteine is attributable not to its amino or carboxyl but to its sulfhydryl group.

TABLE VII

Effect of Cysteine on Thermal Inactivation of GADH

Cysteine[1] (mM)	Preincubation[2]	Heat-treatment	Activity[3]	Per cent residual activity
	(*min.*)			
None	—	—	101	100
None	—	51°, 10 mins.	86	86
20	30	—	110	100
20	30	51°, 10 mins.	99	90
40	30	—	110	100
40	30	51°, 10 mins.	106	96.5
	(*hrs.*)			
None	24	—	112	100
None	24	53°, 7 mins.	46	41.1
20	24	—	110	100
20	24	53°, 7 mins.	94	85.5
40	24	—	105	100
40	24	53°, 7 mins.	110	104.8

1) Freshly prepared before use.
2) Cysteine was preincubated with GADH, 0.002 per cent, at 25°.
3) 0.1 ml. of heated mixture was used to measure activity.

TABLE VIII

Effect of Amino Acids Analogous to Cysteine on Thermal Inactivation of GADH

Additions[1]	Heat-treatment[2]	Activity[3]	Per cent residual activity
None	—	175	100
None	+	130	74.3
DL-alanine	—	178	100
DL-alanine	+	127	71.4
DL-serine	—	175	100
DL-serine	+	130	74.3
DL-aspartate	—	169	100
DL-aspartate	+	140	82.8
L-cysteine	—	189	100
L-cysteine	+	178	94.2

1) Additions, 40 mM. GADH, 0.004 per cent.
2) 51° for 10 minutes.
3) 0.1 ml. of heated mixture was used to measure activity.

DISCUSSION

It is believed that the susceptibility of protein to proteinase depends on

the secondary structure of the protein, because the structure is deformed by denaturation and so becomes more susceptible to proteinase. It has been reported that the susceptibility of cytochrome c (*9*) and hemoglobin (*10*) to proteinase varies according to whether the prosthetic group is in the oxidized or in the reduced state. As reported in the preceding paper (*2*), glutamic acid dehydrogenase was protected from the attack of proteinase or urea by the oxidized form of the coenzyme but not by the reduced form. The latter greatly stimulated the attack. A conception was therefore presented that the secondary structure of glutamic acid dehydrogenase changes depending on whether the coenzyme is in the oxidized or in the reduced state. The present investigation shows that glutamic acid dehydrogenase is also protected from thermal denaturation by DPN^+ but not by DPNH, which greatly accelerates the denaturation process. This result supports the conception described above.

Crystalline glutamic acid dehydrogenase from beef liver has been shown to have a molecular weight of one million at concentration of over 4 mg. per ml. (*11*). Kubo *et al.* (*12*) using the light scattering technique reported that when the enzyme is diluted from over 4 mg. per ml. to less than 1 mg. per ml. the enzyme dissociates into subunits having a molecular weight of about 350,000. Recently, Frieden (*13*) reported that the enzyme, at a concentration of over 4 mg. per ml., dissociates into subunits on additions of DPNH or *o*-phenanthroline but not of DPN^+. The concentration of the enzyme used in his experiments is much higher than that used in our investigation. It is not yet clear whether the subunit is more susceptible to denaturation than the associated molecule. However, the result obtained by Frieden lends some support to the conception described above.

The DPN-CN complex, which has an analogous structure to reduced DPN, also increased thermal inactivation and denaturation of the enzyme. This arouses interest in a comparison of glutamic acid dehydrogenase with papain, since papain is activated by cyanide or reducing agents such as cysteine, thioglycolate, or hydrogen sulfide (*14*).

Reduced DPN was slowly oxidized when incubated with the enzyme protein without substrate. Negelein *et al.* (*5*) suggested that in the reduction of DPN by yeast alcohol dehydrogenase without substrate there might be a transfer of hydrogen from the sulfhydryl group of the protein to the coenzyme. Though the hydrogen transfer has not yet been confirmed for glutamic acid dehydrogenase, if such transfer occurs, increased instability of the enzyme by addition of the reduced coenzyme will be of interest in connection with the properties of sulfhydryl group of the enzyme; for example, the disulfide bond of glutamic acid dehydrogenase may be related to association among the subunits. Conversely the cleavage of this bond by the reductive action of DPNH may bring about dissociation into subunits. However, it has been reported that the disulfide bonds of proteins are reduced by prolonged incubation with a high concentration of cysteine (*4*). It was clearly demonstrated from the effect of cysteine on thermal inactivation of glutamic acid dehydrogenase

however that cysteine did not accelerate the inactivation, but rather protected the enzyme, regardless of the incubation period. It has been shown that denatured protein is easily reduced by cysteine or other reducing agents, leading to an increase in its reactive sulfhydryl groups, but that native protein is difficult to reduce (*15*). From this and the present results, it is doubtful if this dehydrogenase is reduced by cysteine. The mechanism of the protective action of cysteine is as yet obscure. It is, however, conceivable that it may be attributed not to its amino or carboxyl group but to the sulfhydryl group, since other amino acids with structures analogous to cysteine had no protective action.

SUMMARY

Thermal inactivation like inactivation by urea of glutamic acid dehydrogenase prepared from pig liver is depressed by the addition of DPN^+ or glutamate but accelerated by DPNH or the DPN-CN complex.

Unlike alcohol dehydrogenase, DPNH is oxidized by incubation with this enzyme in the absence of substrate. The degree of this oxidation is almost proportional to the amount of the enzyme added.

The enzyme is protected from thermal inactivation by addition of cysteine or hydroxylamine.

The concept that the form of the enzyme protein changes on combination with either DPN^+ or DPNH was discussed.

The author wishes to express his gratitude to Prof. K. Okunuki for his guidance. He would like to thank his colleagues Messrs. K. Iwasaki (Nihon Nosan Kogyo Co., Ltd., Yokohama) and T. Masuda of the same laboratory for helpful discussion during the work.

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The Journal of Biochemistry, Vol. 46, No. 8, 1959

GLUTAMIC ACID FORMATION FROM γ-AMINOBUTYRIC ACID BY BACILLUS PUMILUS. I.

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(Received for publication, December 4, 1958)

Previous studies on the occurrence and distribution of free γ-aminobutyric acid in animal and plant tissues as well as in growing culture media of microorganisms and on the characterization of the enzyme which catalyzes the formation of this amino acid from glutamic acid by decarboxylation have led to an interest in the metabolic fate of γ-aminobutyric acid. Although various evidences for the transamination of γ-aminobutyric acid with α-ketoglutaric acid have been obtained in animal and plant tissues and microorganisms (*1–6*) and some reports on the aerobic metabolism of γ-aminobutyric acid in animal tissues and microorganisms have also appeared (*7–9*), very little is known on the metabolism of the acid.

In our laboratory, a strain of *Bacillus pumilus*, which grew on γ-aminobutyric acid as the sole source of carbon and nitrogen and accumulated a large quantity of L-glutamic acid in its growing culture medium, has been isolated from soil (*10*).

The present paper reports on the metabolism of γ-aminobutyric acid and related substances and on the formation of L-glutamic acid by this strain of *B. pumilus*, 134-a.

METHODS

Cells—Bacillus pumilus 134-a was grown on a shaker at 30° in 500 ml. flasks containing 15 ml. of medium-A with the following composition: γ-aminobutyric acid, 1 per cent; glucose 0.2 per cent; KH_2PO_4, 0.1 per cent; $MgSO_4 \cdot 7H_2O$, 00.2 per cent; pH 7.0; or 30 ml. of medium-B with the additional composition to medium-A: K_2SO_4, 0.1 per cent and Ajinomoto's "Mieki" (aquous solution of amino acid mixture prepared from HCl-hydrolysate of soybean meal, 20 g. of nitrogen per liter), 0.2 per cent. After 24 hours the cells were harvested by centrifugation, washed twice with 0.2 per cent KCl, and suspended in 0.01 *M* phosphate buffer pH 7.0 or 0.2 per cent KCl.

Cell-free Extracts—Washed cells suspended in phosphate buffer were treated with a sonic oscillator (Toyorika Type 50–2, 9 KC, 60–80 w.) for 20 minutes under cooling and the cell debris was removed by centrifugation at 20,000×*g* for 20 minutes at low temperature.

Glutamic acid, γ-aminobutyric acid, glucose, ammonia, and ketoacid were determined respectively by the manometric method using specific L-glutamic decarboxylase of *E. coli* Crookes strain (*11*), by the colorimetric method using the ninhydrin reagent (*11*), by the colorimetric method according to Park and Johnson (*12*), by the

colorimetric method using Nessler's reagent, and by the colorimetric method after extraction of its 2,4-dinitrophenylhydrazone (*13*).

Paperchromatographic Solvent—For amino acids, *n*-butanol-actic acid-water (4 : 1 : 1 or 4 : 1 : 2) and phenol -water (100 : 20) containing final 0.1 per cent NH_3 (*11*); for non-volatile organic acids, *n*-butanol-formic acid-water (4 : 1.5 : 1) (*14*); and for 2,4-dinitrophenylhydrazones of keto acids, *n*-butanol-ethanol-0.5 *N* NH_4OH (7 : 1 : 2) (*15*) and *n*-butanol saturated with 3 per cent NH_3 (*16*) were used. Complete separation of β-formypropionic acid derivative from pyruvic acid, α-ketoglutaric acid and oxalacetic acid derivatives by the following solvents was demonstrated by the following solvents was demonstrated by the present authors as shown in Table I: phenol-water (100 : 20 *v/v*) and *n*-propanol-28 per cent NH_3-water (6 : 3 : 1).

TABLE I

Paperchromatographic Solvents for the Separation of 2,4-Dinitrophenylhydrazone of β-Formylpropionic Acid

Carbonyl compound	R_f-value in paperchromatogram	
	n-Propanol-28% NH_3-water (6 : 3 : 1)	Phenol-water (100 : 20)
β-Formylpropionic acid	0.78	0.84
Pyruvic acid	0.88	0.76, 0.67
α-Ketoglutaric acid	0.68	0.57
Oxalacetic acid	0.68	0.58

2,4-Dinitrophenylhydrazones of above carbonyl compounds were paperchromatographed on Toyo No. 51 paper by ascending method for 15 hours at room temperature (25–30°).

Preparation of β-Formylpropionic Acid—After redistillation of methyl β-formylpropionate *in vacuo*, a solution of free β-formylpropionic acid was prepared by refluxing 0.3 ml. of the ester with 10 ml. of 0.1 *N* HCl for 1 hour. This solution was prepared fresh and neutralized immediately before it was added to the reaction mixture. This solution gave only one 2,4-dinitrophenylhydrazone, which melted at 202–203° (uncorrected) in accordance with Prescott and Waelsch's report (*17*) after twice recrystallization from ethanol.

Determination of N^{15} Content in Glutamic Acid and NH_3 in Medium—After removing the cells by centrifugation and adjusting to pH 7.0, the clear reaction mixture was treated with Amberlite IRA-140-Cl^- column. From this effluent ammonia was distillated into 0.1 *N* H_2SO_4 in vacuo at 60° after the addition of MgO, neutralized 0.1 *N* NaOH, and concentrated to 3–5 mg. N/ml. The eluate by 0.5 *N* HCl from the resin column contained no NH_3 and no ninhydrin positive substances with exception of glutamic acid and its total nitrogen content could be explained completely by its L-glutamic acid content. Glutamic acid was digested according to the Kjeldahl method and ammonia formed was steamdistilled into 0.1 *N* H_2SO_4, neutralized by 0.1 *N* NaOH, concentrated to 3–5 mg. N/ml. After conversion of NH_3 to nitrogen gas by NaOBr, its isotope ratio N^{15}/N^{14} was determined by a Consolidated-Nier Isotope-Ratio Mass-

Spectrometer (CEC 21-201 type) (*18*).

RESULTS

Glutamic Acid Accumulation in Growing Culture Medium—When *B. pumilus* 134-a was aerobically growing in medium-A, L-glutamic acid was accumulated with decrease of γ-aminobutyric acid and glucose, and decreased after the maximum accumulation as shown in Fig. 1. Usually, the degree maximum

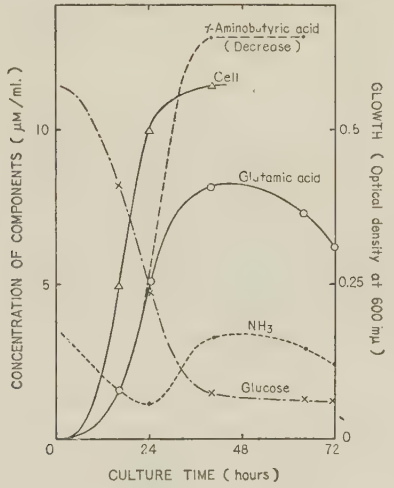

FIG. 1. Accumulation of glutamic acid in culture medium of *B. pumilus* 134-a (aerobic, at 30°).

Culture medium: γ-aminobutyric acid 1 per cent, glucose 0.2 per cent, KH_2PO_4 0.1 per cent, and $MgSO_47H_2O$ 0.02 per cent, pH 7.0.

accumulation of glutamic acid was about 0.3 and 0.5 per cent concentration for medium-A and -B, respectively. Since ammonia was also formed from γ-aminobutyric acid (=γ-ABA), it was of interest whether nitrogen transfer from γ-ABA to glutamic acid occurred *via* ammonia. After the cells grew in medium-A with 0.05 per cent N^{15}-ammonium sulfate, N^{15} concentration of glutamic acid-nitrogen formed and ammonia-nitrogen before and after the cultivation were determined as shown in Table II. That the N^{15} concentration of glutamic acid-nitrogen was lower than that of ammonia-nitrogen after cultivation of cells but higher than that of natural nitrogen suggests the exsistance of both paths of glutamate formation, namely direct transfer of nitrogen from γ-ABA and incorporation of free ammonia after the deamination of γ-ABA. In the latter case, N^{15} concentration of glutamic acid-nitrogen formed should be the same as that of ammonia-nitrogen at that time, which varied from 28 to 8.7 (8.0) per cent during the reaction as shown in Table II.

TABLE II

N^{15}-Concentration of Glutamic Acid-N Formed and NH_3-N Before and After Cultivation

	NH_3 (before) (%)	NH_3 (after) (%)	Glutamic acid (%)
Experiment I.	28.0	8.73	4.37
,, II.	28.0	8.04	3.78
$NH_3 \rightarrow$ glutamic acid	—	—	28~8.7 (8.0) (expected value)
γ-Aminobutyric acid → glutamic acid (direct transfer)	—	—	0.38 (natural)

Culture medium: Medium-A+N^{15}-ammonium sulfate, 0.05 per cent. After shaking at 30° for 54 hours and removing the cells, glutamic acid and NH_3 were separated and converted to N_2 gas, and then its isotope ratio was analysed.

Transamination between γ-Aminobutyric Acid and Glutamic Acid in Cells—The data in Table III demonstrate the transamination reaction that occurred with γ-ABA and α-ketoglutaric acid when intact cell suspension, dried cell suspension, and sonic extracts of *B. pumilus* 134-a were used. Further evidence for this transamination reaction was obtained from the paperchromatographic detection of all reactants and the reverse reaction as shown in Table IV. Specific transaminase activity (Q_T (N)) of the sonic extracts was 1,540 μl./hr., mg. N for the first one hour in the reaction time course. β-Formylpropionic acid formed was identified from paperchromatographic R_f, instability in alkaline solution, and absorption spectrum (Fig. 2) of its 2,4-dinitrophenylhydrazone.

TABLE III

Glutamic Acid Formation from γ-Aminobutyric Acid and α-Ketoglutaric Acid by B. pumilus 134-a cell Preparations

Substrate	Intact cell (0.544 mg. N) (μM/ml.)	Dried cell (0.499 mg. N) (μM/ml.)	Sonic extracts (0.326 mg. N) (μM/ml.)
γ-Aminobutyric acid +α-ketoglutaric acid	9.6	10.7	11.4
γ-Aminobutyric acid	0.2	0.7	0.4
α-Ketoglutaric acid +NH_3	0.5	1.6	1.1
None	0.2	0.5	0.7

Complete system contained enzyme, 50 μM of γ-aminobutyric acid, and 30 μM of α-ketoglutaric acid in a final volume of 1.0 ml. of 0.1 *M* phosphate buffer pH 7.6. Incubated 4 hours at 37°.

Aerobic Metabolism of γ-Aminobutyric Acid—γ-ABA and related substances were metabolized aerobically by intact cell and dried cell suspension as shown

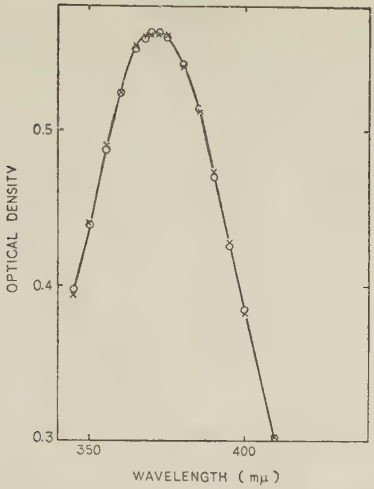

FIG. 2. Absorption spectra of 2,4-dinitrophenylhydrazones of β-formylpropionic acid (—○—) and carbonyl compound (—×—) formed in transamination with γ-aminobutyric acid and α-ketoglutaric acid. Solvent: 0.1 *M* phosphate buffer 7.4.

TABLE IV

Paperchromatographic Evidence of γ-Aminobutyric Acid-Glutamic Acid Transamination Reaction by Sonic Extracts

Substrate	Paperchromatographic detection			
	γ-amino-butyric acid	Glutamic acid	α-Keto-glutaric acid	β-Formyl-propionic acid
γ-Aminobutyric acid+ α-ketoglutaric acid	+	+	+	+
Glutamic acid+ β-formylpropionic acid	+	+	+	+
Glutamic acid	−	+	−	−
γ-Aminobutyric acid	+	−	−	−
α-Ketoglutaric acid	−	−	+	−

Reaction mixture contained sonic extracts 0.118 mg. N, amino acid 50 μM, and 30 μM of carbonyl acid in a final volume of 1.0 ml. of 0.1 *M* phosphate buffer pH 7.6. Incubated 4 hours at 37°. Paperchromatographic solvent: *n*-butanol-acetic acid-water (4 : 1 : 1) for amino acid, *n*-butanol-saturated with 3 per cent NH_3 for 2,4-dinitrophenylhydrazone of carbonyl acid.

in Table V. Resting cells oxidized γ-ABA at a fairly rapid rate, which was affected by various inhibitors as shown in Table VI. There was little change in the rate of oxygen uptake between pH 5.6 and 8.2, suggesting that the

Table V

Respiration rates (Q_{O_2} (N)) of Intact Cell and Dried Cell Suspension of B. pumilus 134-a with Various Substrates

Substrate	Intact cell*	Intact cell**	Dried cell**
γ-Aminobutyric acid	2,540	1,970	600
β-Formypropionic acid	3,070	2,420	—
Succinic acid	110	920	200
Fumaric acid	360	1,500	410
L-Malic acid	980	1,940	435
Oxalacetic acid	210	1,110	130
Pyruvic acid	160	330	75
Citric acid	26	100	59
cis-Aconitic acid	27	190	79
Isocitric acid	100	940	120
α-Ketoglutaric acid	260	610	140
Glutamic acid	920	1,110	400
Glucose	920	970	330
Acetic acid	41	90	16

Culture medium of cells: *, medium-A. **, medium-B.

Reaction mixture contained cell suspension and 100 μM of substrate in 2.5 ml. of 0.1 *M* phosphate buffer pH 7.0, 37°.

reaction possessed a wide optimum pH range.

The complete oxidation of one mole of γ-ABA involves the following reaction:

$$NH_2\text{–}CH_2\text{–}CH_2\text{–}CH_2\text{–}COOH + 4.5\ O_2 = 4\ CO_2 + NH_3 + 3\ H_2O$$

As shown in Fig. 3, the data obtained with *B. pumilus* (O_2: 4.2, CO_2: 3.6 NH_3: 1.0 mole per mole of γ-ABA) suggest that the substrate has been completely oxidized.

Determination of γ-ABA before and after reaction showed that γ-ABA was not metabolized anaerobically by resting cells.

Formation of Pyruvic Acid and L-*Glutamic Acid from γ-Aminobutyric Acid by Resting Cells*—In the oxidation of γ-ABA by resting cells, L-glutamic acid was formed and decreased after the maximum accumulation as shown in Fig. 4. These behaviors of resting cells similar to that of growing cells provide the possibility for the study of the pathway of glutamic acid formation using resting cells.

On the other hand, oxygen absorption stopped at a point of two moles per one mole of γ-ABA in the presence of 5×10^{-3} *M* $NaAsO_2$ as shown in Fig. 3. In this case, evolution of 1.3–1.4 mole of carbon dioxide, formation of 0.98 mole of ammonia, and formation of 0.66 mole of pyruvic acid per mole of γ-ABA were also observed. Evidence that the reaction product was pyruvic acid was provided by the melting point, mix-melting test, absorption

TABLE VI

Effect of Various Substance on the Respiration of Cells with γ-Aminobutyric Acid

Addition	Concentration (*M*)	Degree of inhibition (%)
KCN	0.001	81
KCN+(methylene blue)	,,	72
NaN_3	,,	33
CH_2I-COOH	,,	73
CH^3F-COOH	,,	0
NaF	,,	0
EDTA	,,	93
8-Hydroxyquinoline	,,	91
o-Phenanthroline	,,	100
α, α'-Dipyridyl	,,	37
Nitroso-R salt	,,	−10
$FeCl_3$	,,	−11
$FeSO_4$	,,	−10
$MgSO_4$	,,	−11
$MnSO_4$	,,	−10
$ZnSO_4$	,,	−14
$CaCl_2$	,,	−10
$BaCl_2$	,,	− 4
$CuSO_4$	,,	97
$AgNO_3$	,,	95
$AgNO_3$	0.000001	13
$HgCl_2$	0.0001	96
$FeCl_3$*	0.001	76
$ZnSO_4$*	,,	100
$NaAsO_2$	0.005	15
Semicarbazide	0.001	16.5
Hydroxylamine	,,	94
p-Chloromercuribenzoate	,,	100
Malonate	0.04	0
Malonate	0.2	98

Reaction mixture contained washed cells, 100 μM of γ-aminobutyric acid, and inhibitor above described in 2.5 ml. of 0.1 *M* phosphate buffer pH 7.0 (0.04 *M* Tris buffer pH 7.2 in the case of *), 37°.

spectrum (Fig. 5), and paperchromatographic R_f of its 2,4-dinitrophenylhydrazone, which was recrystallized from water. Glutamic acid, α-ketoglutaric acid, and the other nonvolatile acidic products could not be detected by the paperchromatography of the reaction mixture.

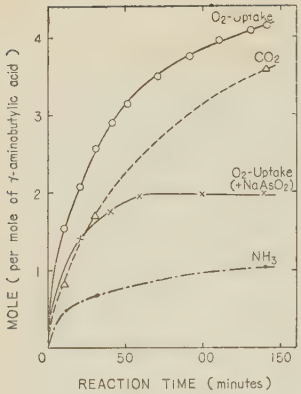

FIG. 3. Oxygen uptake, CO_2 and NH_3 formation, and $NaAsO_2$ inhibition in γ-aminobutyric acid oxidation by cell suspension. Reaction mixture contained cell suspension, 2,5 μM of γ-aminobutyric acid, and 5×10^{-3} M $NaAsO_2$ (in the case of +) in 2.5 ml. of 0.1 M phosphate buffer pH 7.0, 37°.

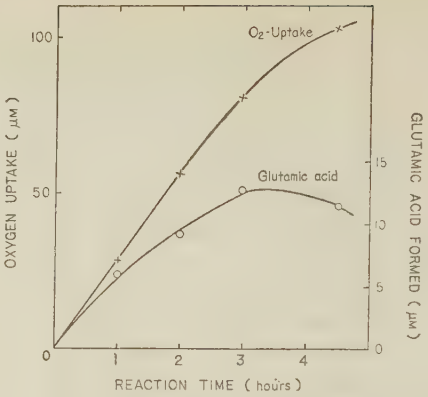

FIG. 4. Oxidation of γ-aminobutyric acid and formation of Glutamic acid by cell suspension. Reaction mixture contained washed cells and 50 μM of γ-aminobutyric acid in 2.5 ml. of 0.04 M phosphate buffer pH 7.0, 37°.

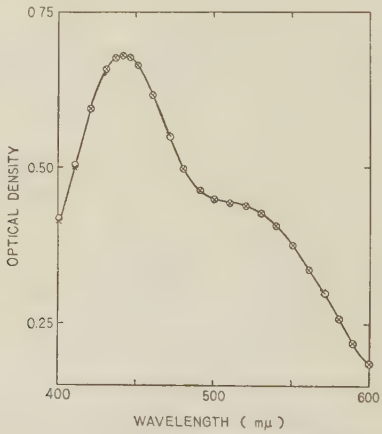

FIG. 5. Absorption spectra of 2,4-dinitrophenylhydrazones of pyruvic acid (—○—) and carbonyl compound (—×—) accumulated in γ-aminobutyric acid oxidation in the presence of $NaAsO_2$. Solvent: 1 N NaOH.

The oxidation of glutamic acid, α-ketoglutaric acid, pyruvic acid, and oxalacetic acid by the same cells was almost completely inhibited by the presence of 5×10^{-3} M $NaAsO_2$ (degree of inhibition calculated from oxygen uptake: 97, 100, 98, and 100 per cent, respectively).

Metabolism of β-Formylpropionic Acid and L-*Glutamic Acid*—β-Formylpropionic acid (βFPA) and its methyl ester were oxidized by resting cells rather at a higher rate than γ-ABA. Metabolic activity for B-FPA was further confirmed

by the decrease of the substrate.

Three and three tenths or more moles of oxygen per mole of β-FPA were absorpted and 5×10^{-3} *M* $NaAsO_2$ inhibited oxygen uptake in the initial phase (62 per cent) and almost completely after about 1.5 moles of oxygen uptake. From the latter reaction mixture, pyruvic acid and a less quantity of α-ketoglutaric acid were detected. No glutamic acid could be detected in the oxidation reaction mixture of β-FPA in the presence of ammonia.

Glutamic acid was metabolized aerobically at a fairly rapid rate but not anaerobically, indicating that the cells had no glutamic decarboxylase activity.

In the oxidation of glutamic acid, oxygen uptake was considerably inhibited by the presence of 5×10^{-3} *M* $NaAsO_2$, 10^{-3} *M* KCN (+ methylene blue), 8-hydroxyquinoline, NH_4OH, ethylenediaminetetraacetic acid, $FeCl_3$, and $ZnSO_4$ (degree of inhibition: 97, 100, 100, 90, 80, 78, and 100, respectively). In the presence of $NaAsO_2$, formation of α-ketoglutaric acid was observed.

Sonic extracts of cells catalyzed the following transamination reactions:

glutamic acid + oxalacetic acid → aspartic acid
glutamic acid + pyruvic acid → alanine
glutamic acid + β-formylpropionic acid ⇄ γ-aminobutyric acid
+ α-ketoglutaric acid (previously described).

DISCUSSION

From the N^{15}-experiment it was suggested that both transamination from γ-aminobutyric acid and reductive amination of α-ketoglutaric acid might be involved in the formation of glutamic acid in the case of growing cells.

The data reported herein indicate, however, that since the cells have an extremely strong transaminase activity with γ-aminobutyric acid and α-ketoglutaric acid the transamination with α-ketoglutaric acid to form glutamic acid may be the chief reaction for the utilization of γ-ABA in the cells and for the formation of glutamic acid, though γ-ABA may be simply deaminated to same extent.

β-Formylpropionic acid formed form γ-ABA *via* transamination reaction was aerobically metabolized at a rapid rate. This fact indicates that this acid may be an intermediate at least in one of the major pathways of metabolism of γ-ABA in the cells.

However the utilization of γ-ABA *via* transamination reaction requires a continuous supply of α-ketogutaric acid as an amino acceptor, which may be performed through the reformation of α-ketoglutaric acid from resulting glutamic acid and the formation *via* TCA-cycle from β-formylpropionic acid, the deamination product of γ-ABA. However, these latter reactions were almost completely inhibited by the presence of arsenite which affected only slightly the initial phase of the oxidation of γ-ABA. In view of these observations and from the fact that there was no activity for anaerobic metabolism of γ-ABA, it was considered that the direct oxidative degradation reaction

might be another major pathway for the utilization of γ-ABA in the cells.

The presence of arsenite caused an accumulation of pyruvic acid, but not of α-ketoglutaric acid, and inhibited completely the formation of glutamic acid and the oxidation of glutamic acid, α-ketoglutaric acid, and pyruvic acid. These facts suggest that the major path of glutamic acid formation may involve the following sequence: γ-aminobutyric acid, β-formylpropionic acid, α-ketoglutaric acid, and glutamic acid. The role of pyruvic acid as an intermediate will be discussed in an subsequent paper.

SUMMARY

1. From the N^{15}-experiment it was suggested that both transamination and reductive amination might be involved in the formation of glutamic acid by *Bacillus pumilus* 134-a.

2. Cell-free extracts of cells showed an extremely strong transaminase activity between γ-aminobutyric acid and glutamic acid ($Q_T(N)=1.540$).

3. It was suggested that the major paths of metabolism of γ-aminobutyric acid might involve the oxidation and the transamination with α-ketoglutaric acid to form glutamic acid.

4. In the course of oxidation of γ-aminobutyric acid by cell suspension, accumulation of glutamic acid and pyruvic acid (in the presence of arsenite) was observed.

5. The data suggest that the major path of glutamic acid formation may involve the following sequence: γ-aminobutyric acid, β-formylpropionic acid, pyruvic acid, α-ketoglutaric acid, and glutamic acid.

The authors are indebted to Prof. S. Akabori, University of Osaka, and Dr. T. Ogawa, Director of our laboratory, for the helpful criticism and encouragement during the course of this work, and to Dr. S. Nishigaki and Mr. K. Kodaira, Institute for Agricultural Technology, the Department of Agriculture and Forestry, for N^{15}-analysis.

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The Journal of Biochemistry, Vol. 46, No. 8, 1959

THE EFFECT OF DETERGENTS ON OXIDATIVE PHOSPHORYLATION IN RAT LIVER MITOCHONDRIA

I. A COMPARATIVE STUDY OF THE EFFECT OF VARIOUS DETERGENTS

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(Received for publication, December 19, 1958)

In the biochemical field attempts have recently been made to use detergents to isolate a submitochondrial fraction capable of oxidative phosphorylation (*1*, *2*) and to prepare cytochrome a with high oxidase activity (*3*). It is possible to use detergents for such preparations. Detergents can also be used in studies on the relation between mitochondrial structure and biochemical function. For this study the detergent is used to disintegrate the mitochondria and so to permit investigation of the resulting changes in biological activity. Various treatments such as hypotonic solutions (*4*), lecithinases (*5*) and phospholipids such as lysolecithin (*6*) had previously been used for disintegration of mitochondria, and detergents were first used by Witter and Mink (*7*). These workers found that nonionic or anionic detergents caused swelling of mitochondria from rat liver. Even at low concentration they caused activation of Mg-activated ATP-ase and inhibition of DNP-activated ATP-ase. On the other hand, cationic detergents such as Cetab did not activate Mg-activated ATP-ase and did not inhibit DNP-activated ATP-ase at low concentration. It was therefore to be expected that cationic detergents such as Cetab and also nonionic and anionic detergents would have effects on oxidative phosphorylation.

The present paper describes the effects of various detergents on oxidative phosphorylation using ascorbate, succinate, β-hydroxybutyrate or α-ketoglutarate as substrates.

MATERIALS AND METHODS

Rat liver mitochondria were prepared according to the method of Hogeboom *et al.* (*8*) with slight modifications, as follows; 0.30 *M* sucrose was used as the preparation medium, and the preparation was centrifuged at $700\times g$ for 4 minutes to remove debris and at $5{,}200\times g$ for 4 minutes to isolate mitochondria. The mitochondrial pellets were kept at 0° in an ice bath before use. The suspension at a concentration equivalent to 3 g. of liver per ml. of suspension was prepared in 0.30 *M* sucrose solution.

From among forty kinds of commercial detergents typical detergents capable of influencing the turbidity of a mitochondrial suspension were selected and used in the present studies. The properties and chemical nature of these are shown in Table I.

TABLE I

Properties of Detergents Used

Trade name	Chemical name or description	Ionic characteristics	Source[1]
Emasol 4130	Polyoxyethylene sorbitan fatty acid ester	none	Kao.
Tween 80	,,	,,	Atlas.
Amilagin	Polyoxyethylene alkylamine	,,	Daiichi.
Aminon 295	,,	,,	Kao.
Neogen TS	Sodium alkylbenzene sulfonate	,,	Daiichi.
Neogen T	,,	,,	Akiyama.
Neugen ES 160	Polyoxyethylene fatty acid ester	,,	Daiichi.
Emanon 1112	,,	,,	Kao.
Digitonon		,,	
Cholate		negative	
MX 655	Polyoxyethylene alkylsodium sulfonyl phenyl ether	,,	Kao.
Amphitol 20 B	Alkyl dimethylammonium acetate	negative and positive	,,
Sanisol C	Alkyl benzyl dimethylammonium chloride	positive	,,
Quatamin 86	Alkyl trimethylammonium chloride	,,	,,
Quatamin 24 P	,,	,,	,,

1) Kao.—Kao Soap Co., Atlas.—Atlas Powder Co., Daiichi—Daiichi Kogyo Seiyaku K. K., Akiyama.—Akiyama Sangyo K. K.,

The effects of the detergents on the stability of mitochondria are expressed in the present paper in terms of transparence and relative concentration. As shown in Table II these correlate with changes in optical density of the mitochondrial suspension. Measurements were made as follows: 0.1 ml. of the detergent was added at the concentrations shown in the upper column of the Table to 0.1 ml. of the original suspension of mitochondria. After standing for 5 minutes the transparence was judged by eye. After standing the change in optical density at 600 mμ was measured on 3.0 ml. of the suspension diluted with 0.30 *M* sucrose solution using a Beckman spectrophotometer. Though the transparence of the suspension was estimated roughly, the results were in good agreement with the values for change in optical density. Therefore, morphological change of mitochondria produced by the detergent has been recorded in terms of transparence. Relative concentration expresses the ratio of concentration of the detergent to that at which the decrease in optical density of mitochondrial suspension starts to occur.

Crystalline ATP*, yeast hexokinase and DPN were prepared as described elsewhere (*9*). Cyt. c was prepared from cow heart (*10*) and used after dialysis against 0.01 *M*

* The following abbreviations are used: ATP, adenosinetriphosphate; DPN, diphosphopyridine nucleotide; cyt. c, cytochrome c; EDTA, ethylenediamine tetraacetate; Pi, inorganic orthophosphate.

Table II

Effect of the Detergents on the Stability of Mitochondria

Detergent	Expression	Initial concentration of detergent (%)										
		0	1×10^{-3}	3×10^{-3}	5×10^{-3}	1×10^{-2}	2×10^{-2}	4×10^{-2}	8×10^{-2}	1.6×10^{-1}	3.1×10^{-1}	6.3×10^{-1}
Quatamin 24 P	Optical density	1.110	1.055	1.080	1.070	0.987	0.514	0.495				
	Transparence		—	—	—	±	+	++				
	Relative concentration		0.1	0.3	0.5	1.0	2.0	4.0				
Emanon 1112	Optical density	0.942						0.920	0.890	0.798	0.614	0.379
	Transparence							—	—	±	+	++
	Relative concentration							0.3	0.5	1.0	2.0	4.0

Conditions are as described in the text.

KCl at 0°. Oxidative phosphorylation of rat liver mitochondria affected by the detergent was measured as follows: the complete reaction system contained (final concentration) 0.002 *M* ATP, 0.01 *M* phosphate buffer, pH 7.0 or 7.4 labeled with P^{32} (10,000 c.p.m. per μ mole of P), 0.005 *M* $MgCl_2$, 0.001 *M* EDTA, 0.01 *M* KF, 0.00002 *M* cyt.c, 0.025 *M* glucose, 1.0 mg. yeast hexokinase, 0.0001 *M* DPN, 0.04 *M* KCl, 0.27 *M* or 0.20 *M* sucrose and 0.01 *M* substrate in a total volume of 1.0 ml. Ascorbate, succinate, β-hydroxybutyrate and α-ketoglutarate were used as substrates. DPN, KCl and 0.20 *M* sucrose were employed in the reaction systems of β-hydroxybutyrate and α-ketoglutarate, and a medium of pH 7.0 was employed only in the case of ascorbate. A hypertonic solution of sucrose was used to inhibit the ATP-Pi exchange reaction (*11*) which disturbs the measurement of oxidative phosphorylation by the isotopic method (*12*). 0.1 ml. of mitochondrial suspension and 0.1 ml. of the detergent were introduced into the side chamber of a Warburg vessel and, after 5 minutes preincubation at 24°, were tipped into the main compartment. Oxygen uptake was measured manometrically at 24° for 20 minutes using trimethylpentane as the manometer fluid (*13*). Assay of phosphorylation was carried out according to the method of L e h n i n g e r *et al.* (*14*).

RESULTS

The results of studies of the effects of various detergents on oxidative phosphorylation in rat liver mitochondria are presented in Table III. It can be seen that with non-ionic, anionic and amphoteric detergents phosphorylation coupled with oxidation of ascorbate-cyt. c is lost at a so low concentration of the detergents that there is no change in transparence of the mitochondrial suspension. With non-ionic detergent such as Emasol-4130, loss of activity occurs at a relative concentration of 0.06. However, the oxidation of ascorbate-cyt. c was markedly accelerated by these detergents. Increased oxidation of cytochrome oxidase in a heart muscle preparation by many detergents was also observed by W a i n i o and A r o n o f f (15). On the other hand, as shown in Table II with cationic detergents such as Sanisol C, Quatamin 86 and Quatamin 24 P, although the decrease in turbidity of a mitochondrial suspension was produced at a lower concentration than that of the other detergents, phosphorylation coupled with oxidation of ascorbate-cyt. c was retained at a relative concentration of the detergents of about 1.0. However, at a high concentration of these detergents, which caused a decrease in turbidity of the mitochondrial suspension, there was a loss of oxidative phosphorylation. Unlike the other detergents, cationic detergents strongly inhibited oxidation of ascorbate-cyt. c in parallel with phosphorylation.

As shown in Table III with all detergents phosphorylation coupled with the oxidation of succinate was retained even at a concentration which caused complete loss of the activity with ascorbate-cyt.c. Although with Neugen ES 160 and Amphitol 20 B, and perhaps also with the other non-ionic and anionic detergents listed in the Table, the loss of oxidative phosphorylation with succinate as substrate occurred at a lower concentration of the detergents than that at which the decrease in turbidity of a mitochondrial suspension started to occur, oxidative phosphorylation with succinate was retained at a relative concentration of 0.2 of Amphitol 20 B and of 0.08 of Neugen ES 160

TABLE III

Effect of Various Detergents on Oxidative Phosphorylation of Rat Liver Mitochondria

Detergent	Initial concentration of detergent (%)	Relative concentration	Experiment number	Transparence	Substrate										
					Ascorbate			Succinate			β-Hydroxybutyrate			None	
					ΔO	ΔP	P/O	ΔO	ΔP	P/O	ΔO	ΔP	P/O	ΔO	ΔP
					(μ atoms)	(μ atoms)		(μ atoms)	(μ atoms)		(μ atoms)	(μ atoms)		(μ atoms)	(μ atoms)
None			I		1.33	1.18	0.89	3.66	5.42	1.48	1.41	3.12	2.21	0.28	0.15
,,			II		2.07	1.06	0.51	1.66	3.32	2.00				0.33	0.34
,,			III		3.45	2.91	0.84	3.70	6.88	1.86				0.49	0.38
Emasol 4130	1×10^{-1}	0.1	I	–	1.33	0.01	—	5.29	2.93	0.56	0.56	0.06	—		
,,	6×10^{-2}	0.06	II	–	3.23	0.03	—								
Tween 80	3	12.0	II	#	4.81	0.01	—							0.31	0.00
,,	5×10^{-2}	0.2	I	–	1.53	0.04	—	4.80	2.07	0.43	0.49	0.06	—		
Amilagin	3×10^{-1}	1.5	II	#	8.22	0.02	—							0.46	0.02
,,	1×10^{-2}	0.05	I	–	1.59	0.60	0.38	3.66	5.46	1.49					
Neogen TS	6×10^{-1}	2.5	II	#	5.54	0.00	—							0.06	0.00
,,	1×10^{-2}	0.04	I	–	1.67	1.20	0.72	3.45	5.19	1.50					
Neugen ES 160	5×10^{-2}	0.4	I	–	1.67	0.00	—	3.60	0.04	—					
,,	1×10^{-2}	0.08	I	–	1.62	0.14	0.09	3.46	1.07	0.31					
Neogen T	2×10^{-1}	1.3	II	#	6.62	0.00	—							0.00	0.00
Aminon 295	2×10^{-1}	1.0	II	±	7.85	0.01	—							0.44	0.02

Detergent	initial concentration of detergent (%)	Relative concentration	Experiment number	Transparence	Substrate										
					Ascorbate			Succinate			β-Hydroxybutyrate			None	
					ΔO	ΔP	P/O	ΔO	ΔP	P/O	ΔO	ΔP	P/O	ΔO	ΔP
Emanon 1112	4×10^{-2}	0.3	II	−	3.38	0.04	—							0.43	0.06
MX 655	5×10^{-1}	1.0	II	±	3.23	0.00	—								
,,	1×10^{-1}	0.2	I	−	1.05	0.03	—	3.95	2.65	0.67	0.56	0.05	—	0.20	0.03
,,	6×10^{-2}	0.1	II	−	2.28	1.09	0.48								
Amphitol 20 B	5×10^{-1}	1.0	II	±	3.62	0.00	—	0.80	0.01	—					
,,	1×10^{-1}	0.2	I	−	1.60	0.10	—	4.01	5.14	1.28				0.36	0.13
,,	6×10^{-2}	0.1	II	−	1.84	0.82	0.45	1.90	3.16	1.66					
Sanisol C	6×10^{-2}	3.7	II	++	0.54	0.02	—								
,,	1×10^{-2}	0.6	I	−	1.33	0.28	0.21	4.65	5.11	1.10	0.99	0.51	0.52	0.36	0.13
,,	4×10^{-3}	0.3	II	−	2.16	0.47	0.22								
Quatamin 86	5×10^{-2}*	5.0	III	++	1.17	0.04	—	2.23	1.30	0.58				0.43	0.03
,,	2×10^{-2}*	2.0	III	+	2.68	0.88	0.33	3.21	4.72	1.47				0.34	0.29
Quatamin 24 P	4×10^{-2}**	4.0	III	++	0.74	0.03	—	0.92	0.04	—				0.11	0.03
,,	3×10^{-2}**	3.0	III	++	0.92	0.06	—	1.83	0.64	0.35				0.12	0.03
,,	2×10^{-2}**	2.0	III	+	1.20	0.12	(0.10)	3.08	1.37	0.45				0.22	0.08
,,	1×10^{-2}**	1.0	III	±	2.88	1.80	0.63	4.97	8.29	1.67				0.29	0.24

* The concentrations are calculated assuming 36% purity of the detergent.

** The concentrations are calculated assuming 28% purity of the detergent. Conditions are as described in the text.

ΔO: Oxygen consumption expressed in μ atoms.

ΔP: Inorganic phosphate incorporated into organic compounds (μ atoms).

(these concentrations caused complete loss of oxidative phosphorylation with ascorbate-cyt. c). With cationic detergents such as Quatamin 86 and Quatamin 24 P, oxidative phosphorylation with succinate remained even at the high relative concentrations which caused aggregation of mitochondria, and inhibition of the ascorbate-cyt. c system.

From those results, it can be assumed that (A) phosphorylation coupled with one step oxidation at the level of cytochrome oxidase is specifically inhibited by the detergents but that the other step in the transport of electrons from succinate to oxygen is not affected so much, (B) though each of these two steps in the complete electron transport system retains some activity, coupled of the ascorbate-cyt. c system to phosphorylation at the level of cytochrome oxidase is inhibited by the detergents. To confirm these assumptions, a comparison was made between the P:O volues of phosphorylation coupled with oxidation of ascorbate-cyt. c and succinate in the presence of cholate or Quatamin 24 P. As shown in Table IV, though oxidative phosphorylation with ascorbate-cyt. c was completely lost at a low concentration of

TABLE IV

Effect of Concentration of Cholate on Oxidative Phosphorylation with Ascorbate or Succinate as Substrate

Initial concentration of detergent	Relative concentration	Transparence	Substrate					
			Ascorbate			Succinate		
			ΔO	ΔP	P/O	ΔO	ΔP	P/O
(%)			(*μ atoms*)	(*μ atoms*)		(*μ atoms*)	(*μ atoms*)	
0			1.33	1.18	0.89	2.34	4.24	1.81
1.3×10^{-2}	0.2	−				2.86	5.11	1.79
2.5×10^{-2}	0.3	−				3.26	6.03	1.85
5.0×10^{-2}	0.7	−	1.53	0.03	—	3.19	6.01	1.88
7.5×10^{-2}	1.0	±				2.04	3.10	1.52
1.5×10^{-1}	2.0	+	1.63	0.14	—	1.78	2.54	1.43
2.5×10^{-1}	3.3	+				2.10	0.04	—

Conditions are as described in the text.

cholate, the activity with succinate was retained at the relatively high concentration which caused a change in transparence of mitochondrial suspension. Also it was markedly increased in proportion to increment in the concentration of detergent which did not cause the change in transparence of the suspension. Moreover, it was observed that the P:O value for oxidative phosphorylation with succinate was maintained at 1.88 and did not drop to the value of the control (1.81) at a relative concentration of 0.7 in spite of complete loss of phosphorylation coupled with oxidation of ascorbate-cyt. c. It can be seen in Table III that with a relative concentration of 0.2 of Amphitol 20 B the P:O value of oxidative phosphorylation with succinate is maintained at 1.28 in spite of complete loss of activity of the ascorbate-cyt. c

Table V

Effect of the Concentration of Quatamin 24 P on the Oxidation Phosphorylation

Initial concentration of detergent	Relative concentration	Transparence	Substrate													
			Ascorbate			Succinate			β-Hydroxybutyrate			α-Ketoglutarate			None	
			ΔO	ΔP	P/O	ΔO	ΔP	P/O	ΔO	Δ/P	P/O	ΔO	Δ/P	P/O	ΔO	ΔP
(%)			(μ atoms)	(μ atoms)		(μ atoms)	(μ atoms)		(μ atoms)	(μ atoms)		(μ atoms)	(μ atoms)		(μ atoms)	(μ atoms)
0			4.42	1.99	0.45	3.86	5.30	1.37	1.48	2.69	1.82*	1.76	4.70	2.67	0.18	0.24
1×10^{-3}	0.1	−	4.55	1.66	0.37	4.20	5.85	1.39							0.18	0.09
4×10^{-3}	0.4	−				4.18	6.29	1.50	1.54	2.21	1.44	1.52	4.07	2.68	0.18	0.11
7×10^{-3}	0.7	−	3.56	1.02	0.29	5.25	7.37	1.40	1.54	1.69	1.10	1.79	4.58	2.56	0.17	0.03
1.0×10^{-2}	1.0	±	3.14	0.85	0.27	5.53	7.98	1.44				1.89	4.04	2.13	0.11	0.01
1.4×10^{-2}	1.4	+	1.83	0.42	0.23	5.15	3.76	0.73	0.78	1.02	1.31	1.95	2.89	1.48		
2.1×10^{-2}	2.1	+	1.04	0.06	—	3.40	0.39	0.11	0.80	0.87	1.09	2.05	1.48	0.72		
2.4×10^{-2}	2.4	++	0.75	0.06	—	3.22	0.28	0.09				2.01	0.80	0.40		
2.8×10^{-2}	2.8	++	0.74	0.03	—	3.06	0.22	0.07	0.84	0.52	0.62	2.14	1.07	0.50		
3.4×10^{-2}	3.4	++	0.64	0.04	—	1.38	0.06	—				0.96	0.42	0.44	0.06	0.03

Conditions are as described in the text.

* The low P : O value was owing to hypotonic conditions (0.15 *M* sucrose) of the mitochondrial suspension during preincubation, since 2.23 of the value was obtained in a suspension in 0.30 *M* sucrose.

system. A definite increase in oxidative phosphorylation was also observed with Quatamin 24 P as shown in Table V. Further studies on the increase in activity with these detergents will be described in the following paper (*16*). Table V shows that in proportion to increase in concentration of Quatamin 24 P to a level which did not cause a change in transparence of the mitochondrial suspension, the activity of phosphorylation coupled with oxidation of succinate is greatly increased while the P:O value stays as higher as that of the control. However there is a decrease in activity and lowering of the P:O ratio with ascorbate-cyt. c. Therefore it is concluded that a detergent such as cholate, Amphitol 20 B or Quatamin 24 P does not inhibit phosphorylation coupled with a specific individual step in the transport of electrons from substrate to oxygen but does inhibit the coupling of the ascorbate-cyt. c system with phosphorylation at the level of cytochrome oxidase.

Marked inhibition of oxidation of succinate was observed at a high relative concentration of detergents such as Quatamin 86, Quatamin 24 P and Amphitol 20 B. Inhibition by Amphitol 20 B was observed in oxidation of succinate but uot with ascorbate-cyt. c as shown in Table III. However cationic detergents strongly inhibited both of oxidation of succinate and ascorbate-cyt. c. Inhibition of oxidation by Quatamin 24 P may be attributed to denaturation of mitochondrial cytochrome a and b. These were studied with handspectroscope (Table VI). It was found that the appeared in 2 and 4 hours, respectively, at 30° at a relative concentration of 4.2 of the detergent

TABLE VI

Effect of Quatamin 24 P on Cytochromes of Mitochondria

Incubation (*hours*)	Cytochrome a			Cytochrome b			Cytochrome c		
	Concentration of Quatamin 24 P ($\times 10^{-1}$%)								
	0	2.8	8.3	0	2.8	8.3	0	2.8	8.3
0	++			+++			+++		
1	++	+	+	+++	+++	++	+++	+++	++
2	++	+	±	+++	+++	+	+++	+++	++
3	++	±	−	+++	++	±	+++	+++	++
4	++	±	−	+++	++	±	+++	+++	++

The detergent was added to a suspension of mitochondria equivalent to 2.0 g. of liver. 2.8×10^{-1} and 8.3×10^{1} per cent final concentration correspond to 4.2 and 8.3 relative concentration of the detergent respectively. The measurement was carried out with a hand-spectroscope.

and in 1 and 2 hours at a relative concentration of 12.4. After treatment with detergents the absorption bands of the pyridine-haemochromogens of these cytochromes were normal. Denaturation of cytochrome a and b was also

Table VII

Effect of Concentration of Digitonin on Oxidative Phosphorylation

Initial concentration of detergent	Relative concentration	Transparence	Substrate										
			Ascorbate			Succinate			β-Hydroxybutyrate			None	
			ΔO	ΔP	P/O	ΔO	ΔP	P/O	ΔO	ΔP	P/O	ΔO	ΔP
(%)			(μ atoms)	(μ atoms)		(μ atoms)	(μ atoms)		(μ atoms)	(μ atoms)		(μ atoms)	(μ atoms)
0			1.32	0.83	0.63	2.40	3.50	1.46	2.47	5.33	2.16	0.44	0.16
5×10^{-2}	0.3	−	1.14	0.69	0.61	2.12	2.70	1.27	2.54	2.66	1.05	0.49	0.13
1.5×10^{-1}	1.0	±	1.20	0.27	0.23	2.82	1.84	0.65	2.73	1.13	0.41		
2.5×10^{-1}	1.7	+	1.25	0.08	—	2.47	1.20	0.49	2.48	0.14	—	0.42	0.05
3.5×10^{-1}	2.3	+	1.20	0.06	—	1.13	0.28	0.25	2.16	0.07	—		
5.0×10^{-1}	3.3	++	1.29	0.06	—	1.13	0.10	—	1.78	0.10	—	0.45	0.04
7.5×10^{-1}	5.0	++	1.46	0.07	—	1.13	0.11	—	0.64	0.00	—		
1.0	6.7	++	1.59	0.08	—	1.06	0.10	—				0.58	0.02

Conditions are as described in the text.

observed in Keilin-Hartree preparations (*17*) treated with Quatamin 24 P and Amphitol 20 B but not with Emasol 4130.

Oxidation of β-hydroxybutyrate or α-ketoglutarate and coupled phosphorylation were strongly suppressed at low concentrations of all non-ionic, anionic and amphoteric detergents. However, with cationic detergents such as Quatamin 24 P, these activities were retained even at high concentrations which caused aggregation and change in transparence of the mitochondrial suspension. Lehninger *et al.* (*1*), reported that detergents such as digitonin were used for isolation of a submitochondrial fraction which was capable of phosphorylation coupled with oxidation of β-hydroxybutyrate and ascorbate-cyt. c and of oxidizing succinate. However, Table VII shows that digitonin suppressed the oxidation and coupled phosphorylation with β-hydroxybutyrate as strongly as that with ascorbate-cyt. c. Oxidation and coupled phosphorylation with succinate was rather less inhibited as in the case of all other detergents. This unexpected result may be attributed to inhibition by the detergents added to the reaction medium.

DISCUSSION

It has been reported that addition of cyt. c is necessary for oxidation of ascorbate by cytochrome oxidase in intact mitochondria (*18*, *19*), and that phosphorylation coupled with oxidation of ascorbate-cyt. c is markedly increased by slight hypotonic treatment of intact mitochondria (*19*, *20*). It is considered that the increase in activity by this treatment is due to facilitation of approach of the externally added ascorbate-cyt. c system to the active site of cytochrome oxidase in the mitochondria. However, it is an interesting problem why ascorbate itself, which has a much smaller molecule than cyt. c is unable to approach the active site of the oxidase. Prevention of approach of ascorbate to the site of the oxidase cannot be due to impermeability of the mitochondrial surface membrane, since the submitochondrial particles isolated with digitonin (*21*) or sonic vibration (*22*, *23*) also need externally added cyt. c for oxidation of ascorbate by their particulate cytochrome oxidase. Though ascorbate is a common reducing agent, in the fraction capable of oxidative phosphorylation it seems to be unable to reduce directly internal cytochrome c functioning as an electron donor for cytochrome oxidase. Therefore, there is no reason why a mechanism should not exist which prevents the transport of electrons directly from ascorbate to internal cytochrome c but which allows electron transport from ascorbate to internal cytochrome c through externally added cytochrome c. In the latter case, it may also be possible that external cytochrome c reduced by ascorbate functions directly as an electron donor for cytochrome oxidase.

Table IV showes that at a relative concentration of cholate of 0.7 the P : O value of oxidative phosphorylation with succinate as substrate remained at 1.88 in spite of complete loss of the activity of the ascorbate-cyt. c system. Therefore it is concluded that the detergent does not inhibit phosphorylation coupled with a specific individual step at the level of cytochrome

oxidase in the electron transport system, but at that level inhibits coupling of the ascorbate-cyt. c system with the phosphorylation. In consequence, it may be that the mechanism which is capable of transporting the electrons from ascorbate through externally added cytochrome c to the electron donor for cytochrome oxidase, plays the further role of coupling the externally added ascorbate-cyt. c system to phosphorylation at the level of cytochrome oxidase. Detergents, such as cholate, damage the coupling mechanism but have no effect on phosphorylation at the level of cytochrome oxidase. Consequently oxidation of ascorbate-cyt. c which is not inhibited by a low concentration of detergent proceeds through the cytochrome oxidase shunt.

Lardy *et al.* (*22*) and Kielley *et al.* (*23*) observed that in a submitochondrial fraction prepared by sonic vibration the P:O value of oxidative phosphorylation coupled with the oxidation of succinate by oxygen was less than 1.0. Therefore an attempt was made to determine whether the loss of oxidative phosphorylation occurred at a step or steps in the transport of electrons from succinate to oxygen. Lardy *et al.* found that phosphorylation coupled with the oxidation systems of succinate-ferricytochrome c or ferricyanide and the ascorbate-cyt. c system was not detected, on the contrary, Kielley *et al.* found that the P:2e value agreed well with the value for the succinate-oxygen system only in the succinate-ferricytochrome c system, though the activity was low. Witter *et al.* (*6*) working with mitochondria treated with lysolecithin found that though phosphorylation coupled with oxidation of succinate by oxygen had a P:O value of nearly 1.0, neither the individual succinate-ferricyanide or ascorbate-cyt. c system could be detected. Low P:O values for oxidation of succinate by oxygen were also observed in the present experiments. Table III shows that in liver mitochondria treated with the detergents phosphorylations coupled with oxidation of succinate proceeded, although at a concentration of the detergents which caused complete loss of phosphorylation coupled to oxidation of ascorbate-cyt. c, the P:O values were less than 1.0. This partial loss of the activity seems to be due to inhibition of a step at the level of cytochrome oxidase. However, by analogy with the effects observed with detergents such as cholate, Amphitol 20 B and Quatamin 24 P it may be that loss of phosphorylation with ascorbate-cyt. c results from inhibition of coupling of the ascorbate-cyt. c system with the phosphorylation. If phosphorylation can be inhibited either at a step in the succinate-ferricytochrome c system or in the ascorbate-cyt. c system, it probably occurs in the former. This assumption is contrary to the results of Kielley *et al.* and is based on the apprehension of Chance and Williams (*24*). These latter workers apprehended that, in the heart muscle preparation of Slater and Holton (*25*), a P:O values of about 3 for oxidation of α-ketoglutarate to succinate and of about 1 for oxidation of succinate by oxygen were due to inactivation of cytochrome b of the preparation.

The coupling mechanism of the ascorbate-cytochrome c system with phosphorylation seems to be unstable. This is suggested by the loss of activity which occurs when the mitochondria are slightly aged even when the P:O

value for oxidation of succinate by oxygen is sometimes more than 1.

SUMMARY

1. A comparative study has made with mitochondria of effects of various detergents on phosphorylation coupled with oxidation of ascorbate-cyt. c, succinate, β-hydroxybutyrate-DPN and α-ketoglutarate.

2. Non-ionic, anionic and amphoteric detergents inhibited oxidative phosphorylation with these substrates at a low concentration which did not cause a change in transparence of mitochondrial suspension. Cationic detergents however inhibited oxidative phosphorylation of these substrates less at a even relatively high concentration which caused aggregation of mitochondria.

3. With all detergents used in the present study, phosphorylation coupled with oxidation of succinate was retained even at a concentration of detergent which completely inhibited the ascorbate-cyt. c system.

4. With cholate and Amphitol 20 B, not only was retained oxidative phosphorylation with succinate at a concentration which caused complete loss of the activity with ascorbate-cyt. c, but also the P : O value was maintained at more than 1.

5. From the result, it is assumed that inhibition of oxidative phosphorylation by the detergent using ascorbate-cyt. c as substrate is due to uncoupling of the ascorbate-cyt. c system from phosphorylation at a step at the level of cytochrome oxidase.

6. Oxidative phosphorylation coupled with oxidation of succinate was markedly increased at a low relative concentration of cholate or Quatamin 24 P.

The author wishes to express his thanks to Prof. K. Okunuki, Mr. K. Iwasa and T. Higashiyama for their kind advice during this work, and to the Kao Soap Co. Ltd., Daiichi Kogyo Seiyaku K. K., and Akiyama Sangyo K. K. for gifts of detergents.

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The Journal of Biochemistry, Vol. 46, No. 8, 1959

CONSIDERATIONS ON THE POSSIBILITY OF APPARENTLY UNI-DIRECTIONAL CATALYSIS, WITH SPECIAL REFERENCE TO FISCHER AND EYSENBACH'S "FUMARIC REDUCTASE"

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(Received for publication, December 20, 1958)

In connection with a discussion made between Dr. Singer (*1*) and the writer at the International Symposium on Enzyme Chemistry, in Tokyo (1957), the present article is intended to give a brief account of a tentative mechanism for explaining the apparent paradox involved in the reaction of "*Fumaric reductase*" of F. G. Fischer and Eysenbach 1937 (*2*).

The enzyme was originally described by the German authors to catalyze the interconversion of succinate and fumarate exclusively in the direction of reduction of fumaric to succinic acid, without showing any evidence of activity towards oxidation (dehydrogenation) of succinic to fumaric. The existence of such an enzyme with an exclusively uni-directional capacity for activation, taken alone, seemed to the writer to be in contradiction with the general notion of catalysis, especially since the reaction system concerned was a reversible one with a definite level of oxidation-reduction potential. The circumstance, however, that the enzyme in question had been supposed by the original authors to be a flavoprotein undergoing a cycle of oxidation and reduction in the course of catalysis led the present writer to the following consideration on the subject, which he published in preliminary form (*3*) in Japanese in colaboration with Dr. H. Tamiya who had arrived at the same idea, independently, in his investigations on the reaction of cytochromes in living cells.

Let us assume the following as the most simple scheme of reaction involved in the catalysis in question;

$$\text{(i)}\quad F+EH_2 \underset{k_1'}{\overset{k_1}{\rightleftarrows}} F\cdot EH_2 \underset{k_2'}{\overset{k_2}{\rightleftarrows}} S+E, \qquad (K_1=k_1'/k_1;\ K_2=k_2/k_2')$$

$$\text{(ii)}\quad F+E \underset{k_3'}{\overset{k_3}{\rightleftarrows}} F\cdot E \qquad (K_3=k_3'/k_3)$$

$$\text{(iii)}\quad F\cdot E+AH_2 \underset{k_4'}{\overset{k_4}{\rightleftarrows}} F\cdot EH_2+A \qquad (K_4=k_4'/k_4)$$

where F and S stand, respectively, for fumaric and succinic acids; E and EH_2 are the enzyme—actually a flavoprotein—in oxidized and reduced forms,

respectively, of which the combined states with the substrates are denoted by $F \cdot E$ and $F \cdot EH_2$; the hydrogen acceptor and its reduced form are expressed as A and AH_2, respectively. The k's represent the velocity constants of the reaction steps indicated; the K's are the equilibrium constants to be derived from those reaction constants.

Assuming a rapid reaction between the oxido-reductive dye and the enzyme (Equation iii) and considering the fact that the rate of reaction (v) in F. G. Fischer's paper was shown to be maximal at a fumarate concentration as low as $10^{-4}\,M$, we may infer that the rate-determining step of the over-all reaction was the one indicated with k_2 in the above scheme (Equation i),

$$v = k_2\,[F \cdot EH_2] \tag{1}$$

At the beginning stage of the reaction, the reverse reaction (Step k_2') can safely be neglected because of the negligibly small amount of the reaction product, succinic acid, S. Under the condition of saturation with respect to the fumarate concentration (see above), the bulk of the enzyme is supposed to be in combination with the substrate, no significant amounts of E and EH_2 remaining in the reaction mixture. The following calculation will then apply as regards the relative concentrations of the two forms of enzyme-substrate combination to be considered.

$$\frac{[F \cdot E]\,[AH_2]}{[F \cdot EH_2]\,[A]} = K_4 \tag{2}$$

Let us denote the equilibrium constants for the oxidation-reduction of the enzyme and the dye by $K_{(E)}$ and $K_{(A)}$, respectively;

$$K_{(E)} = \frac{[E]\,[H]^2}{[EH_2]}, \qquad K_{(A)} = \frac{[A]\,[H]^2}{[AH_2]} \tag{3 and 4}.$$

If we rewrite Equation 2 using these constants, the following relationship will be obtained;

$$K_4 = \frac{K_1 \cdot K_{(E)}}{K_3 \cdot K_{(A)}} \tag{5}$$

Therefore, under the conditions of the reaction postulated above, the ratio of $[F \cdot EH_2]$ to $[F \cdot E]$ or to ε, the total concentration of the enzyme, will be as follows;

$$\frac{[F \cdot EH_2]}{[F \cdot E]} = \frac{[AH_2] \cdot K_3 \cdot K_{(A)}}{[A] \cdot K_1 \cdot K_{(E)}} \tag{6}$$

or,

$$\frac{[F \cdot EH_2]}{\varepsilon} = \frac{1}{1 + \dfrac{[A] \cdot K_1 \cdot K_{(E)}}{[AH_2] \cdot K_3 \cdot K_{(A)}}} \tag{6'}$$

where, ε is the total concentration of the enzyme, which is equal to the sum of $[F \cdot EH_2]$ and $[F \cdot E]$ so far as the condition of calculation we have assumed is concerned.

Now, if we have before us an enzyme with significantly lower potential as compared with that of the dye, *i.e.*,

$$E_{0'(E)} < E_{0'(A)}$$

and consequently, in Equations 3 and 4,

$$K_{(E)} > K_{(A)},$$

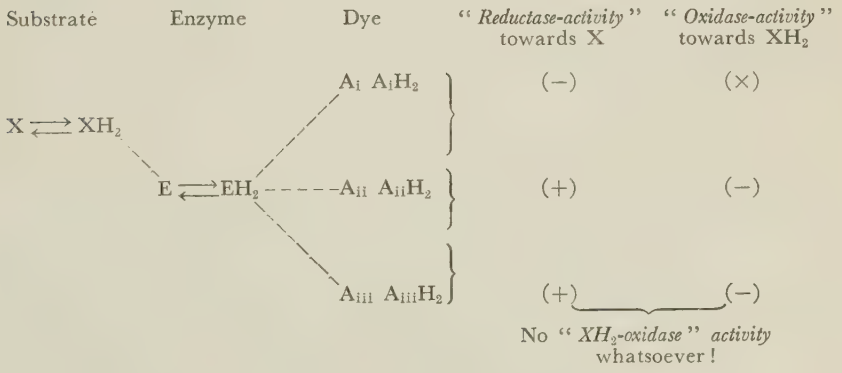
then it follows that, with a given ratio of $[AH_2]/[A]$, the concentration $[F \cdot EH_2]$ and hence, the reaction rate v (see Equation 1) will become smaller with the decrease of the relative level of $E_0'_{(E)}$ as compared with $E_0'_{(A)}$.

To cite examples from the results of F. G. Fischer *et al.*, the rates of enzymatic re-oxidation of the reduced form of the dye (in the presence of saturating amounts of fumaric acid) was fairly large with dyes with lower levels of potential (*e.g.*, Janus green, E_0'; -0.251 and methylene violet, E_0'; -2.256), complete re-oxidation of AH_2 taking place only in 20 minutes. With Nile blue having an E_0' of -0.150, the reaction was much slower (complete re-oxidation in 12 hours). With dyes situated still higher in the oxidation-reduction scale, the re-oxidation did not proceed to completion. The re-oxidation was reported to cease practically completely at 70-80 per cent and 20 per cent re-oxidation of the reduced forms of indigo-disulfonate and indigo-trisulfonate, respectively. (Indigo-disulfonate, E_0'; -0.125 and -trisulfonate, E_0'; -0.080). With dyes of higher redox-potential, there was no appreciable re-oxidation of the substance added, although the level of E_0' concerned was not so high as to make the over-all reaction impossible from the thermodynamic point of view.

Estimation made according to Equation 6′ indicates that a difference of

$$E_0'_{(A)} - E_0'_{(E)} = +0.08 \text{ (volt)}$$

will satisfactorily account for the observed fact that the reaction rate of the



X and XH_2; the substrate and its reduced form.

(×); no reaction because of the circumstance discussed above.

(−); no reaction ever possible from thermodynamic grounds.

(+); positive catalysis.

(Oxidation-reduction systems involved are arranged in the above table according to their level of potential E_0').

catalytic re-oxidation dropped—at 20 per cent oxidation of the dye—to 1/100 that of the maximal capacity of the enzyme (Equation 6′) (see the above results with indigo-trisulfonate). A similar calculation* for indigo-disulfonate with a slightly lower value for $E_0'_{(A)}$ will readily show that the re-oxidation can proceed a little further (70–80 per cent in Fischer's experimental results; see above before the greater portion of the enzyme assumes the F · E form according to Equation 6′.

The writer is not unaware of the lack, for the present, of substantial proof of his theory, but he thinks this line of inference might be of some interest in that it accounts for the possible existence of a "reductase" -type of enzyme practically incapable of catalyzing the reaction in the reverse direction. The general view concerning this *paradox of "reductase"* may be presented in the scheme in the foregoing page.

In conclusion, it may be stated that the above line of argument will also apply in the considerations on the possibility of the existence of apparently uni-directional "oxidase" -type of enzyme.

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* In this calculation, for the sake of simplicity, identical values of K_1 and K_3 were assumed.

The Journal of Biochemistry, Vol. 46, No. 8, 1959

STUDIES ON NITRATE REDUCTASE SYSTEM OF ESCHERICHIA COLI

I. PARTICULATE ELECTRON TRANSPORT SYSTEM TO NITRATE AND ITS SOLUBILIZATION

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In a series of studies on nitrate reductase of *Escherichia coli*, Egami and Sato revealed the sulfhydryl and metalloenzyme nature of the NaR and its mode of action in the electron transport system for nitrate reduction (*1–4*). They found that FAD* and cyt. b_1 were functioning as intermediary electron carriers. It was the first finding of cytochrome in an anaerobic electron transfer. Nitrate reduction such as found in *E. coli* has been called "nitate respiration", because of its physiological and enzymic similarity to oxygen respiration (*5*, *6*).

Further purification of the enzyme was carried out in order to elucidate the mechanism of nitrate reduction. Cell-free enzyme preparation extracted from *E. coli* cells, however, showed a remarkable resistance to various purification procedures such as salt or organic solvent fractionation, isoelectric point precipitation, gel adsorption and zone electrophoresis in a starch column, suggesting that NaR of *E. coli* might be localized in a cellular particle.

The present paper will report on the particulate nature of NaR and the electron transport system to nitrate in *E. coli* together with their modification with isobutanol and some detergents by which particulate NaR is solubilized.

MATERIALS AND METHODS

Microorganisms—E. coli (Yamaguchi strain) was grown on the peptone-broth-agar plate containing 0.1 per cent KNO_3 at pH 7.0 for 15 to 17 hours at 37°. The cells were harvested and washed three times by centrifugation.

Disruption of Bacterial Cells by Ultrasonic Vibration and by Incubation with a Concentrated Solution of Glycine—The washed cells of 15 to 20 g. in 75 ml. of distilled water and exposed to the ultrasonic vibration (560 KC) for 35 minutes at room temperature and then centrifuged at $3,500 \times g$ for 15 minutes to obtain a cell-free extract as the supernatant.

* The following abbreviations are used: DPNH, reduced diphosphopyridine nucleotide; FAD, flavin adenine dinucleotide; EDTA, ethylenediamine tetraacetic acid; PCMB, *p*-chloromercuribenzoate; Mb, methylene blue; MbH_2, reduced methylene blue; cyt., cytochrome; NaR, nitrate reductase.

Cells of some bacteria including *E. coli* has been known to be disrupted by incubating in concentrated solutions of α-amino acids. (*7*) *E. coli* cells were disrupted by a glycine solution as described by Gordon (*7*). The cells were suspended in 2 to 3 *M* glycine solution containing 0.05 *M* phosphate buffer, pH 7.5 at the same concentration of cells as employed in the ultrasonic disruption. The suspension was incubated for 15 to 17 hours at 37° to disrupt the cells.

Fractional Centrifugation of the Cell-free Extracts—The cell-free extracts prepared were centrifuged stepwisely at 10,000, 20,000, and 110,000$\times g$ for 20, 40 and 40 minutes, respectively, using a International centrifuge and a Spinco model L ultracentrifuge at below 5°. Four fractions termed as $P_{3\cdot5-10}$*, P_{10-20}, P_{20-110}, and S_{110} were obtained. For the studies on the electron transport system and the effects of metal-binding inhibitors as well as on the solubilization, the particulate fraction $P_{3\cdot5-20}$ was used.

Procedure for Solubilization of the Particulate NaR by Treatment with Isobutanol or Some Detergents—The $P_{3\cdot5-20}$ fraction was suspended in 0.05 *M* acetate buffer, pH 4.8 containing 1×10^{-3} *M* EDTA, then subjected to the freezing and thawing two or three times repeatedly. The particles so modified as to be more easily sedimentable could be collected by centrifugation at 15,000$\times g$ for 15 minutes and resuspended in 0.05 *M* Tris buffer, pH 8.9 containing 1×10^{-3} *M* EDTA and KNO_3. 0.6 volume of isobutanol cooled at $-10°$ was added slowly to one volume of the suspension and stirred at 0° in an ice-water bath for 30 minutes. The emulsion formed was centrifuged at 20,000$\times g$ for 40 minutes to separate into three layers: isobutanol (upper), aqueous (middle) and precipitate (lower) one. The aqueous layer was collected and used as a solubilized preparation.

The particulate preparation was treated to solubilize by some detergents such as deoxycholate, according to the method described by Wainio (*8*). The aliquot of 10 per cent solution of a detergent was added to the particulate fraction suspended in 0.025 *M* phosphate buffer, pH 7.0 and homogenized by a glass homogenizer and allowed to stand in an ice-cold bath for at least two hours. Then the suspension was centrifuged to obtain the supernatant as a solubilized preparation.

Enzyme Assay:

Enzyme activity was assayed anaerobically in a Thunberg tube at 37° after 10 minutes preincubation.

Nitrate Reductase—NaR activity was assayed using MbH_2 as an electron donor as described previously (*9*). The reaction mixture contained 0.025 *M* phosphate buffer, pH 6.8, 7.5, μ moles of MbH_2, 10 μ moles of KNO_3 and enzyme preparation in a final volume of 5 ml. KNO_3 in a side room was tipped into the main room to start reaction. In this paper, the term "nitrate reductase" was used only to refer to the electron-transferring activity from MbH_2 to nitrate assayed as described here.

Formate-NaR System—"Formate-NaR system" was termed for the system participating in the electron transfer from formate to nitrate in this paper. Its activity was assayed using formate as a hydrogen donor without adding any artificial intermediary carrier such as Mb. The reaction mixture was the same as above except that MbH_2 was replaced by 50 μ moles sodium formate. Nitrate and formate in a side room were tipped into the main room to start the reaction.

DPNH-NaR System—"DPNH-NaR system" was termed for the system participating in the electron-transfer from DPNH to nitrate in this paper. Its activity was assayed spectrophotometrically using a Thunberg tube-type cuvette at room temperature, (15°

* $P_{3\cdot5-10}$, *etc*, refer to the particulate fraction obtained between 3,500 and 10,000$\times g$, *etc*. S_{110} refers to the supernatant at 110,000$\times g$.

to 20°). Oxygen had to be removed perfectly from the reaction mixture, since the enzyme preparation contained active aerobic DPNH oxidase as shown later. The reaction mixture contained 0.025 *M* phosphate buffer, pH 6.8, 0.15-0.3 μ mole of DPNH, 10 μ moles of KNO_3 and the enzyme preparation in a final volume of 3.0-3.5 ml. Nitrate in a side room was tipped into the cuvette compartment to start the reaction and decrease in the optical density at 340 mμ was followed by a Hitachi spectrophotometer.

Formic and Lactic Dehydrogenase—The activities of these dehydrogenase were assayed by decolorization of Mb. Reaction mixture contained 0.025 *M* phosphate buffer, pH 6.8, 50 μ moles of sodium formate or sodium lactate, 0.3 μ mole of Mb and the enzyme preparation.

Units and Specific Activities of NaR, NaR System and Dehydrogenase—One unit of NaR system was defined as the amount of enzyme required to produce 1.0 μ mole of nitrite from nitrate per hour under each assay condition. One unit of dehydrogenase was defined as the amount of enzyme required to reduce 1.0 μ mole of Mb per hour. Specific activities of these were expressed as units per mg. of Kjeldahl nitrogen in enzyme preparation.

Determination of Protoheme—The protoheme of cyt. b_1 was extracted from enzyme preparation by 30 per cent pyridine solution in 0.1 *N* NaOH. Almost all of the protoheme could be extracted as pyridine hemochromogen by this procedure, as scarcely any amount of protoheme was detected in the residue of extraction. Optical densities of pyridine hemochromogen thus prepared were measured at 408 mμ in the oxidized state and at 435 and 557 mμ in the reduced state. The protoheme was estimated from the three standard curves determined for the pure protoheme preparation from bovine erythrocyte*. The amount of protoheme was expressed as the mean value of those three data which always coincided well with each other indicating no appreciable contamination of other hemes.

RESULTS

Particulate Nature of the Nitrate Reductase System

Distribution of NaR System—The ultrasonic extracts were fractionated by the differential centrifugation. As shown in Table I, more than 80 per cent of the original NaR activity was recovered in the three particulate fractions ($P_{3.5-10}$, P_{10-20}, P_{20-110}) with three to four times higher specific activity. On the contrary, less than 10 per cent of the original activity and much lower specific activity were found in the soluble fraction (S_{110}). Formic dehydrogenase activity and the amount of protoheme were distributed essentially parallel with the NaR activity, as shown in the same table. Lactic dehydrogenase activity, though not listed, was also found in the particulate fractions. The similar distribution pattern of NaR was obtained when the isotonic sucrose solution (0.25 *M*) was employed as the preparation medium.

The results show that NaR as well as formic and lactic dehydrogenase and cyt. b_1 (protoheme) are all localized in the particulate fractions, suggesting the residence of these four oxido-reduction enzyme on a common particle.

* For the data the authors are indebted to Miss H. Kumada of the Department of Biology, Nagoya University.

One can not exclude the possibility, however, that the particles prepared might be modified from the natural state or even be artifacts derived from some insoluble cellular materials.

TABLE I

Distribution of Formic Dehydrogenase, Protoheme and NaR in the Ultrasonic Extract of E. coli Cells

Fraction	Total nitrogen (*mg.*)	Formic dehydrogenase		Protoheme		NaR	
		Total activity (*unit*)	Specific activity (*unit*/*mg.* N)	Total amount (*μg.*)	Concentration (*μg.*/*mg.* N ×100)	Total activity (*unit*)	Specific activity (*unit*/*mg.* N)
Original extract	98	1000	10	0.21	0.21	1600	16
$P_{3.5-10}$	14	200	15	0.07	0.50	550	39
P_{10-20}	8.2	500	60	0.10	0.85	350	45
P_{20-110}	22	250	12	0.10	0.45	600	27
S_{110}	62	60	1	0.06	0.10	80	1.3

Original extract was obtained as the supernatant of ultrasonic extract at 3,500×*g*. For further details see text.

TABLE II

Effect of Ultrasonic Treatment on the Particulate Preparation

	Protein N (*mg.*)	NaR	
		Total activity (*unit*)	Specific activity (*unit*/*mg.* N)
Original particulate preparation	—	410	—
After the second treatment	13.5	440	33
Fraction S_{20} after the second treatment	5.0	190	38
Fraction S_{110} after the second treatment	2.1	45	20

To investigate the disrupting effect of ultrasonics in this connection, the particulate preparation ($P_{3.5-20}$) was again exposed to the ultrasonics and fractionated in a similar way. The results are shown in Table II. After the second treatment, a significant increase of NaR activity was observed in the smaller particulate fraction (P_{20-110}) and soluble fraction (S_{110}). The specific activity of NaR in the two fractions was found to be comparable with those

of the original larger particulate preparation (Table I). Thus the fraction $P_{3.5-20}$ was shown to be fragmented by ultrasonic treatment into smaller particles and soluble materials.

TABLE III

Distribution of NaR in Cellular Fractions of Glycine Extract

	Recovery per cent		
	Crude extract	S_{20}	S_{110}
Protein	100	50	—
NaR activity	100	1	0

Disruption of E. coli Cells dy Concentrated Glycine Solution—The disruption pattern of NaR in the cell-free extracts prepared by glycine treatment was different from that observed in the ultrasonic extracts. As shown in Table III, 50 per cent of the protein (Folin method) of the cells was extracted indicating the disruption of the cells, whereas no traces of NaR, formic dehydrogenase and cyt. b_1 were found in the supernatant fraction S_{20}. Furthermore, it was observed that the ultracentrifugation of fraction S_{20} gave no sediments.

It may be suggested from these results that NaR, formic dehydrogenase and cyt. b_1 were localized in the large particles or some insoluble cellular materials derived by glycine treatment.

Electron Transport System to Nitrate in the Particulate Preparation

The Functioning of Cyt. b_1 and the Pathway of Electron Transfer from MbH_2—As mentioned above, NaR, formic dehydrogenase and cyt. b_1 are all localized in the particulate fractions. Concerning their interrelation in the electron transfer from formate to nitrate, Sato *et al.* revealed that cyt b_1 which had been reduced enzymatically by formate was reoxidized anaerobically by the addition of nitrate using ultrasonic extract form *E. coli* (*4*). Further evidence for the participation of cyt. b_1 in the formate- and DPNH-NaR system was obtained by the aid of a specific inhibitor of cyt b and b_1, 2-heptyl-4-hydroxy-quinoline-*N*-oxide, which had been shown by Lightbown *et al.* to inhibit the electron transport system involving cyt. b or b_1 of mammals and some microbes (*10*). The quinoline-*N*-oxide inhibitor produced 70 to 80 per cent inhibition for both formate and OPNH-NaR activities and 20 to 30 per cent activities remained even in the saturated inhibition (Figs. 1 and 2). NaR activity assayed by MbH_2 was inhibited only 10 per cent by similar level of the quinoline-*N*-oxide. The aerobic oxidation of DPNH by the particulate preparation having a much higher rate than that of the oxidation by nitrate was also sensitive to the quinoline-*N*-oxide to the same extent. Both formic and DPNH dehydrogenases were shown to be insensitive to the quinoline-*N*-oxide.

It is evident that cyt. b_1 cannot be involved in the electron transfer from MbH_2 to nitrate but in those from formate or DPNH to nitrate and from DPNH (or probably formate) to molecular oxygen.

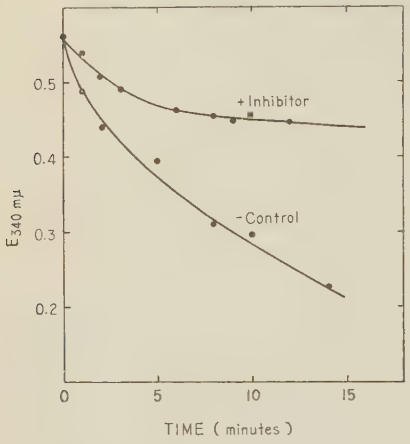


FIG. 1. Effect of quinoline-*N*-oxide on DPNH-NaR system.

0.25 μ mole DPNH and 0.015 μ mole the quinoline-*N*-oxide were added to 3.5 ml. of reactionmixture. When no inhibitor was added, 0.2 μ mole nitrite produced enzymatically was determined after the reaction.

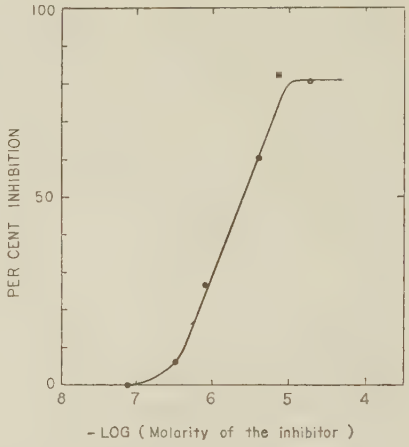


FIG. 2. Effect of the quinoline-*N*-oxide on formate-NaR system.

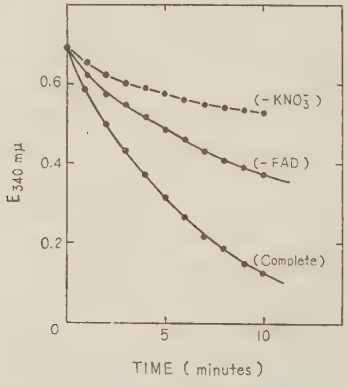


FIG. 3. Effect of FAD on DPNH-NaR system.

Complete system contained 3.6×10^{-3} μ moles FAD, 0.3 μ mole DPNH, 10 μ moles KNO_3, " soluble activator " and the particulate preparation. For further details see text. In complete system, nitrite of the same amount as DPNH oxidized was produced.

The Effect of FAD and Other Factors on Formate- and DPNH-NaR System—It was already revealed by Egami *et al.* that chemically reduced FAD was an effective hydrogen donor for nitrate reduction and that FAD could participate as an intermediary hydrogen carrier in a reconstructed NaR system (*3*). As shown in Fig. 3, the externally added FAD accelerated the oxidation of DPNH by nitrate in the particulate preparation, suggesting the participation of some flavoprotein in DPNH-NaR system. On the contrary, formate-NaR system was not accelerated by the external FAD.

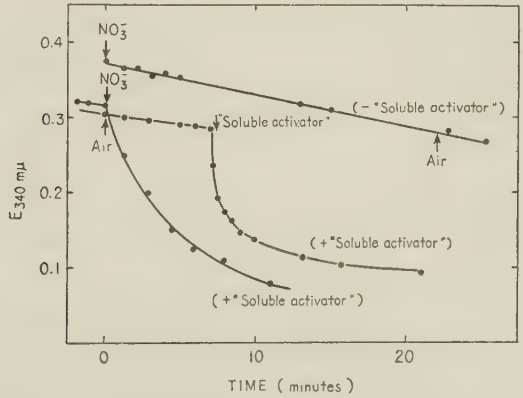
The rate of the electron transfer observed in the particulate preparation from DPNH or formate to nitrate via cyt. b_1 was shown to be less (20–30 per cent) than that form MbH_2. In this connection, the soluble fraction (S_{110}) was found to activate not only DPNH- or formate-NaR but the aerobic

TABLE IV

Effect of "Soluble Activator" on Formate-NaR System

Particulate Preparation used (*ml.*)	Electron donor		
	Formate		MbH_2
	No "soluble activator	With "soluble activator	No "soluble activator
0.25	0.9	3.0	4.0
0.50	2.2	5.4	7.3
1.00	4.4	12.0	12.6

The figures represent μ moles of nitrite produced in 20 minutes.



Effect of "soluble activator" on the oxidation of DPNH by nitrate and oxygen.

Complete system contained 0.15 μ mole DPNH and 0.25 ml. "soluble activator". Other components same as in text.

oxidation of DPNH by the particulate preparation. The activation was also demonstrated by the centrifugal supernatant (10,000×*g* 15 minutes) of the boiled (90, 20 minutes) ultrasonic extracts (Table IV and Fig. 4.). Table IV shows that the activity of formate-NaR system was accelerated to the same level as that NaR assayed by MbH_2.

The soluble fraction was found to act neither as an electron donor for NaR nor as an activator for NaR and formic dehydrogenase. Even the high level of FAD, ferrous ion and menadione (alone or in combination) preincubated with the particulate preparation did scarcely replace the function of the fraction. The soluble activator in this fraction described here was found to be heat stable, dialyzable, acid labile and resistant against the action of a crystalline protease from *B. subtilis**.

Effect of Metal-binding Inhibitors on NaR—As summarized in Table V, cyanide and azide were potent inhibitors, whereas EDTA, 8-hydroxyquinoline and CO even in the dark scarcely inhibited the enzyme. Thiourea, o-phenanthroline and α, α'-dipyridyl inhibited NaR to the extent of 50 to 70 per cent at 10^{-3} *M*. CO inhibition in the dark was less than that previously reported by Sato *et al.*, who claimed iron for the metallic component of NaR (*4*).

Table V

Effect of Inhibitors on NaR

Inhibitor	Per cent inhibition			
	Final molarity			
	10^{-2}	10^{-3}	10^{-4}	10^{-5}
KCN		100	95	70
NaN_3		100	90	
α, α'-Dipyridyl[1]		40[1]		
o-Phenanthroline		50		
8-Hydroxyquinoline[2]		5[2]		
EDTA		0		
Hydroxylamine		0		
Thiourea	80	70		
KCNO		70		
PCMB[1]		70[1]		
CO (dark)	1 *atm.* 10			

1) Final molarity was 4×10^{-3} *M*.
2) Final molarity was 2×10^{-3} *M*.

Preincubation of the enzyme preparation with metallic ions such as Fe^{++}, Fe^{+++}, Mn^{++}, MoO_4^{--}, Zn^{++}, Cu^{++}, Co^{++}, Ni^{++}, Mg^{++}, and WO_4^{--}, at 5×10^{-4} *M* final concentration did not affect the NaR activity. Though NaR was

* The authors thank to Nagase Co Ltd. for the gift of this crystalline enzyme.

inactivated to half or one third of the original activity by dialysis against KCN-phosphate buffer solution, the inactivated portion was not restored by the addition of various metallic ions as mentioned above. A similar result was obtained by cyanide dialysis for soluble NaR prepared by isobutanol treatment.

Solubilization of the Particulate NaR System

Solubilization of the Particulate NaR System—To obtain the soluble NaR, the particulate preparation was treated by acetone, *n*-butanol, isobutanol, cholate, deoxycholate, steapsin, trypsin and a protease prepared from *B. subtills* singly or in various combinations. Isobutanol treatment was found to be the most effective method among these trials. NaR and formic dehydrogenase were found only in an aqueous fraction produced by the isobutanol treatment. After removing isobutanol by dialysis, the aqueous fraction was centrifuged at $80{,}000 \times g$ for 40 minutes. This high speed supernatant contained almost all of the original activities indicating that the two enzyme were solubilized (Table VI). The recovery of soluble NaR from the particulate source was 30 to 50 per cent (the enzyme was partially inactivated by the treatment), and the specific activity of the soluble NaR was 1.6 times higher than that of the particulate NaR. Replacing isobutanol with *n*-butanol, the complete inactivation of NaR occurred during the treatment.

Table VI

Solubilization of the Particulate Preparation by Isobutanol

	Fraction	NaR		Formic dehydrogenase	
		Total activity (*unit*)	Specific activity (*unit/mg.* N)	Total activity (*unit*)	Specific activity (*unit/mg.* N)
Exp. 1.	Original particulate preparation	400	31	490	38
	S_{20} after treatment	220 (55%)	38	440 (90%)	85
	P_{20} ,,	0	—	0	—
Exp. 2.	S_{20} after treatment	26.4	40		
	S_{80} ,,	22.0 (90%)	60		

Concerning the formic dehydrogenase insensitive to the isobutanol treatment, two to three-fold increase in its specific activity was attained.

Some Properties of the Soluble NaR System—The absorption spectra of the soluble NaR preparation showed the presence of cyt. b_1 and the ratio of cyt. b_1 to NaR activity was one-half of that found in the particulate preparation. The spectrum in ultraviolet region showed a peak at 276 mμ, whereas the

characteristic absorption spectrum of nucleoprotein was observed for the particulate preparation. The activities of both formate- and DPNH-NaR were completely abolished and not restored by FAD or the soluble activator described above, though formic and DPNH dehydrogenase activities (assayed in the presence of Mb as well as of NaR activity remained in the soluble preparation*.

The storage of the soluble NaR caused 50 per cent inactivation at pH 4.8 for 2 hours and 30 to 50 per cent inactivation at pH 7.0 for 24 hours in the cold (0–5°). On the contrary, the activity of the particulate NaR was quite stable under these conditions.

Table VII shows that deoxycholate extracted NaR activity from the particulate preparation, which remained in the supernatant after ultracentrifugation. The particulate materials seemed to be dispersed and prevented from sedimenting by the presence of deoxycholate, because any trial of complete removal of deoxycholate has not been attained without producing insoluble materials having NaR activity. The presence of some level of deoxycholate seems to be necessary to maintain the soluble state.

Replacement of deoxycholate with cholate in the range of 0.5 to 4.0 per cent gave a less effect on the solubilization. Application of dodecyl sulfate (0.5 per cent in final concentration) completely inactivated NaR of the particulate preparation.

Table VII

Solubilization of the Particulate Preparation by Deoxycholate

Fraction	NaR	
	Total activity (*unit*)	Recovery (*per cent*)
Original particulate preparation	240	
S_{20} after treatment	180	75
S_{20} after treatment	140	
S_{110} ,,	125	90

The particulate preparation was treated with deoxycholate in a final concentration of 4 per cent.

The activity of the soluble NaR prepared by the deoxycholate treatment was abolished by the action of trypsin or a protease from *B. subtills*, unlike that of the particulate NaR.

* It was, however, that chemically reduced cyt. b_1 (with dithionite) is able to reduce nitrate in the presence of the soluble NaR, while DPNH or formate is unable to reduce cyt. b_1. It seems, therefore, that a factor functionally connecting formic or DPNH dehydrogenase with cyt. b_1 was broken by the isobutanol treatment.

Moreover, deoxycholate itself was found to exhibit inhibitory effects on the electron transport system of the particulate NaR system. Formate-NaR activity was remarkably inhibited by 10^{-4} *M* deoxycholate, while either NaR or formic dehydrogenase activity was far less sensitive towards the same level of deoxycholate (Table VIII). When the formate-NaR activity was assayed in the presence of Mb, the inhibition by deoxycholate fell to the same extent as it was the case with the NaR activity. The results indicate that deoxycholate inhibits not the individual enzymes but rather their interactions in the particulate formate-NaR system functionally connecting them.

TABLE VIII

Effect of Deoxycholate on NaR and Formate-NaR System

Enzyme system	Per cent inhibition			
	Final molarity of deoxycholate			
	2.5×10^{-2}	5×10^{-3}	10^{-3}	1×10^{-4}
NaR[1]	50	10	—	(+10)
Formate-NaR	98	95	40	12
Formate-Mb-NaR[2]	56	10	—	—

1) NaR activity was assayed using MbH_2 as an electron donor. For further details see text.

2) Mb was added at 5×10^{-4} *M* to the reaction mixture of the assay for formate-NaR.

DISCUSSION

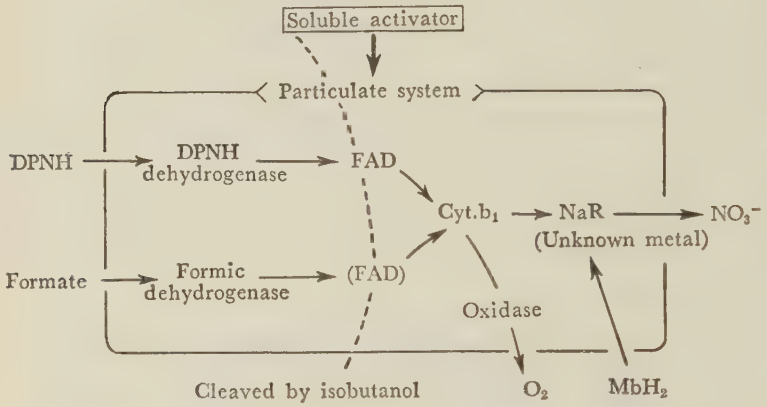
Recently, most of microbial oxido-reduction enzymes were shown to take particulate forms as in the cases of mammalian tissues (*11*, *12*). The nitrate-reducing system of *E. coli* involving NaR, cyt. b_1 and DPNH, formic and lactic dehydrogenases was demonstrated to reside on a particulate cell-structure. This fact presents an evidence of its close similarity to respiratory enzyme systems in various tissues. Weibull obtained a sedimentable cellular fragment named "ghost" by means of lysozyme and hypotonic treatment possessing respiratory systems including cytochromes, and suggested that bacterial particles corresponding to mammalian mitochondria resided on the "ghost" (*13*). Unlike the enzyme localization in the smaller particulate fraction of the ultrasonic extracts, the larger sedimentable cellular fragments similar to "ghost", though not morphologically investigated, could be obtained by the glycine treatment as an only fraction containing NaR, cyt. b_1 and formic dehydrogenase. Ultrasonic treatment probably not so mild as either Weibull's or glycine treatment seems to break the cellular structure into smallar fragments rather continuously.

The particulate form of NaR system appears to be a predominant one, since the activity of the soluble NaR produced by ultrasonic treatment was

considerably lowered and glycine treatment did not give any soluble form of NaR, though no further discusssion on the intactness or homogeneity of such particles can be permitted here.

The particulate electron transport not only of formate-NaR already characterized by Sato *et al.* (*4*) but of DPNH-NaR and DPNH oxidase were shown to have cyt. b_1 participation. By application of a quinoline-*N*-oxide inhibitor, it was concluded that MbH_2 could donate its electrons directly to NaR. The whole sequence can be summarized by the following scheme:

Particulate Nitrate Reductase System of E. coli



The fact that the quinoline-*N*-oxide inhibition remains at 70 to 80 per cent and never reaches 100 per cent even in saturated level may suggest that a quinoline-*N*-oxide insensitive NaR system not involving cyt. b_1 is responsible for 20 to 30 per cent of the activity of NaR system. The simultaneous occurrence of two different types of NaR system in *E. coli* was previously discussed (*6*, *14*).

The experiments were carried out to clarify the relationship between the quinoline-*N*-oxide and naphtoquinone derivative in the NaR system. The latter were found by Wainwright (*15*) to be essential factors of TPNH-NaR system of *E. coli*. Menadione neither stimulated the activity of formate-NaR system nor restored the inhibition of fomate-NaR system caused by the quinoline-*N*-oxide. On the contrary, $10^{-3}\,M$ of menadione restored partially (50 per cent) the inhibition of formate-NaR system caused by $2\times10^{-4}\,M$ of dicumarol, which perfectly inhibited formate-NaR system at $5\times10^{-3}\,M$. Therefore, it seems possible that a certain naphtoquinone derivative participates in the NaR system in addition to such factors as flavin or cytochrome and that the site attacked by quinoline-*N*-oxide is different from the naphtoquinone site.

The finding of a soluble activator, though not chemically identified, which enhanced formate- and DPNH-NaR in the particulate preparation presents an example of cooperation of particulate system with a soluble factor. Such

a typical pattern of organized electron transport systems has also been found in the microbial oxidative phosphrylation system (*16*). The activator seems to be presumably of non-protein and low molecular nature. The function of the activator could not be replaced by the preincubation with FAD, menadione and ferrous ion. The latter were reported by Wainwright to function in TPNH-NaR system of *E. coli* (*15*). The determination of the site as well as of chemical nature of the activator remains to be studied.

Cytochrome involvement in the NaR system has extensively been revealed in halotolerant Micrococcus No. 203*, some denitrifyers and Achromobacter as in *E. coli* (*17*, *18*). In contrast with the NaR participating in the nitrate assimilation of soluble and molybdoflavoprotein nature (*19*) (*e.g.*, TPNH-NaR in *Neurospora*), the cytochrome involvement as well as the particulate nature of NaR system seem to be characteristic of the system of nitrate respiration type. It seems desirable to investigate further the interrelation between cytochrome and NaR.

It is of interest that both isobutanol and deoxycholate not only structurally modify the particulate NaR system to the soluble state but also simultaneously block the interrelation among each enzymes. Similar effect of deoxycholate on DPNH oxidase or succinoxidase was reported by Mackler (*20*) and Kalman (*21*). Isobutanol which blocks somewhere between formic or DPNH hehydrogenase and cyt. b_1 seems to be similar to isooctane, by which the particulate DPNH-cyt. c-reductase of rat muscle was inhibited (*22*). Deoxycholate which disperses the particulate NaR system in a soluble state seems to bring about some denaturation, considering the appearence of the lability of NaR toward a protease action.

SUMMARY

1. NaR, cyt. b_1, formic and DPNH dehydrogenases were shown to be localized in the particulate fractions in the ultrasonic extracts from *E. coli*, suggesting the residence of these four enzyme on a common particulate structure forming the particulate NaR system.

2. In contrast with ultrasonic treatment, glycine treatment of *E. coli* cells yielded none of the smaller particulate preparation, but the larger insoluble cellular fragments, on which NaR, cyt. b_1 and formic dehydrogenase resided.

3. The whole sequence of electron transfer in the particulate NaR system was represented by a scheme. The enzymic electron transfer from MbH_2 to nitrate was insensitive towards 2-heptyl-4-hydroxyquinoline-*N*-oxide, indicating no participating of cyt. b_1, in contrast with formate- or DPNH-NaR which was sensitive towards the inhibitor. The possibility of the participation, though not dominant, of a quinoline-*N*-oxide insensitive mechanism not involving cyt. b_1 was discussed.

4. Not only DPNH- or formate-NaR but aerobic DPNH oxidase in the particulate preparation was found to be activated by an acid labile soluble ac-

* Unpublished observation in our laboratory.

tivator presumably of non protein and low molecular nature. The function of the soluble activator could not be replaced by FAD, menadione and ferrous ion.

5. Menadione did not restore the inhibitory effect of quinoline-*N*-oxide on the NaR system but that of dicumarol.

6. Some heavy metal component was suggested to be tightly bound in NaR from the inhibitory action of cyanide and azide.

7. Isobutanol treatment caused solubilization of NaR as well as cyt. b_1, DPNH and formic dehydrogenase together with simultaneous blocking of some intermediary step(s) between DPNH or formic dehydrogenase and cyt, b_1 in the particulate NaR system.

8. Deoxycholate was found to disperse the particulate NaR system to a solube state and inhibited formate-NaR system almost completely at 5×10^{-3} *M* final concentration.

The authors wish to express their gratitude to Prof. Egami for his useful and encouraging advice, and to the staff of the research-group of "Inorganic nitrogen metabolism" for their assistance and discussions throughout this investigation. The authors also express their thanks to Prof. Fukai of Institute for Microbial Desease, Osaka University and to Dr. Yamamoto of the Department of Microbiology, Nagoya, University for operating Spinco-ultracentrifuging apparatus.

This research was supported in part by a grant from the Scientific Research Fund of the Ministry of Education.

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The Journal of Biochemistry, Vol. 46, No. 8, 1959

STUDIEN ÜBER DIE METHYLIERUNG DER PYRIDIN-VERBINDUNGEN IM TIERE-ORGANISMUS

IV. PAPIERCHROMATOGRAPHISCHE UNTERSUCHUNGEN ÜBER DAS VERHALTEN DER NICOTINSÄURE IM KANINCHEN-ORGANISMUS*

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(Der Schriftleitung zugegangen am 7. Januar 1959)

Vor mehreren Jahren hat D. Ackermann (*1*) eine Beobachtung gemacht, dass Nicotinsäure, welche Hunden per os eingeführt war, als zwei umgewandelte Verbindungen d. i. Nicotinursäure und Trigonellin im Harn ausgeschieden wird.

Einerseits, um diese interessante Beobachtung von Ackermann nachzuprüfen, und andererseits, um den intermediären Stoffwechsel der Nicotinsäure bei verschiedenen Tierarten zu vergleichen, haben später Y. Komori und Y. Sendju (*2*) eine Reihe von Fütterungsversuchen mit Nicotinsäure an Hunden und Kaninchen angestellt. Dabei wird im Einklang mit der Annahme Ackermanns die am Hunde verabreichte Nicotinsäure zum Teil nach Paarung mit Glykokoll als Nicotinursäure und zum Teil unter Methylierung und Betainierung als Trigonellin ausgeschieden, während bei Kaninchen die Säure nur an Glykokoll gekuppelt wird und als Trigonellin nicht zum Ausscheidung gelangt.

Es wäre merkwürdig, dass das Kaninchen, in dessen Harne nach Eingabe von Pyridin die entsprechende *N*-Methylverbindung in sehr geringer Menge im Vergleich zu anderen Tierarten ausgeschieden wird, Nicotinsäure nicht zu methylieren vermöge. Ich erinnere mich der Versuche, die von E. Abderhalden (*3*) und M. Tomita (*4*) ausgeführt wurden. Abderhalden's Experimente zeigten, dass das Kaninchen nicht befähigt sei, das aufgenommene Pyridin in die Methylverbindung überzuführen, während das Pyridin im Organismus des Hundes eine Methylengruppe aufnimmt und als Methylpyridylammoniumhydroxyd in den Harn übergeht. Im Gegenteil dazu hat Tomita festgestellt, dass der Organismus des Kaninchens auch imstande sei, das gefütterte Pyridin zu methylieren, und wenn auch ein quantitativer Unterschied zwischen beiden Tierarten bestehe, im Prinzipe die chemische Organisation nicht verschieden sei.

Es muss also noch dahingestellt bleiben, ob der Kaninchen-Organismus absolut ausserstande sei, die Nicotinsäure in die *N*-Methylverbindung überzu-

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führen; denn es wäre auch denkbar, dass die Menge des gebildeten Umwandlungsproduktes zu gering ist, als dass man es nachweisen könnte.

Auf Veranlassung von Prof. M. Tomita habe ich mir die Frage vorgelegt, ob die angebliche Differenz im Methylierungsvermögen der Nicotinsäure bei Kaninchen und anderen Tierarten nicht qualitativer, sondern nur quantitativer Natur sei.

Zur Entscheidung dieser Frage wurden einige papierchromatographischen Untersuchungen an Kaninchen angestellt, deren Resultate im folgenden mitgeteilt werden.

EXPERIMENTELLE BELEGE

Vier mittelgrosse Kaninchen wurden zum Versuch benutzt. 2.5 g. Nicotinsäure wurden an einen Kaninchen innerlich verabreicht. Nach der Fütterung der Nicotinsäure wurde der Harn jeden Tag gesammelt, der sofort *in vacuo* bei 55–60° eingedampft und mit dem Alkohol extrahiert. Die alkoholischen Auszüge wurden abgedampft und dann in 2 ml. Aceton gelöst. 0.01–0.04 ml. dieser Acetonlösung wurde zur chromatographischen Untersuchung benutzt. Eindimensional und aufsteigend mit *n*-Butanol: Aceton: Wasser = 45 : 5 : 50 auf Papier Nr. 50 oder 51 der Toyo-Filtrierpapier- Gesellschaft gearbeitet; Laufzeit 13 Stunden bei 20°.

TABLE I

Papierchromatogramme der Nicotinursäure und des Trigonellins

Lösungsmittel	Nicotinursäure		Trigonellin	
	R_f-Wert	Farbe des Flecks	R_f-Wert	Farbe des Flecks
n-Butanol, Aceton und Wasser 45 : 5 : 50 (*v/v*)	0.13	Rot	0.07	Gelb

Zum Indizieren der Chromatogramme ist von mir ein folgendes *p*-Aminobenzoesäure-Verfahren vorgeschlagen worden. Zunächst werden getrocknete Chromatogramme mit der 0.5 prozent Aethanollösung von *N*-Naphthylaethylendiamin-2HCl befeuchtet. Nach dem Trocknen werden sie dann mit der Lösung (*5*) (2 g. *p*-Aminobenzoesäure, in 75 ml. 0.75 *N* HCl gelöst und auf 100 ml. mit 90 prozent Aethanol aufgefüllt) befeuchtet. Nach dem Trocknen des Papiers können die einzelnen Flecke der Papierchromatogramme durch 1 stündiges Beräuchern in CNBr Atmosphäre beobachtet werden.

Es ergab 2 Flecke, nämlich ein roter Fleck bei dem R_f-Wert 0.13 und ein gelber Fleck bei dem R_f-Wert 0.07 (Fig. 1).

Diese R_f-Werte entsprechen denen der Nicotinursäure und des Trigonellins nach der Angabe von Johnson (*6*). Mit den Kristallen von Nicotinursäure und Trigonellin, die durch Fütterungsversuche mit Nicotinsäure an Hunden aus Harn dargestellt worden sind, wurde ihre R_f-Werte in denselben Beding-

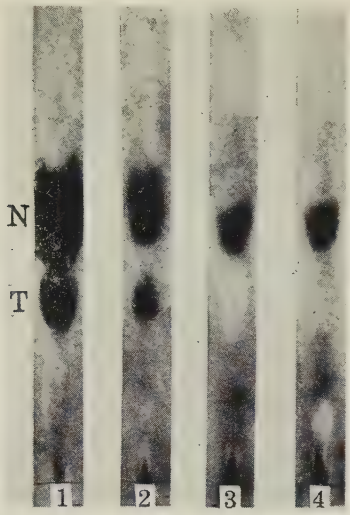

FIG. 1. Flecks; N: Nicotinursäure, T: Trigonellin.
1: 1 Tag nach der Fütterung, 2: 2 Tage nach der Fütterung,
3: 3 Tage nach der Fütterung, 4: 4 Tage nach der Fütterung von Nicotinsäure.

ungen ermittelt. Man erhielt dabei die mit den Johnson's Angaben übereinstimmenden Werte.

ZUSAMMENFASSUNG

1. Die vorliegende papierchromatographische Untersuchung hat als Umwandlungsprodukte der Nicotinsäure im Kaninchenorganismus Nicotinursäure und Trigonellin ergeben.

2. Das Kaninchen ist also befähigt, wenn auch in geringeren Mengen, verfütterte Nicotinsäure in die Methylverbindung überzuführen.

3. Trotz sorgfältigen Suchens nach Nicotinsäureamid wurde es doch nicht gefunden.

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The Journal of Biochemistry, Vol. 46, No. 8 1959

TURBIDOMETRIC TITRATION OF SERUM PROTEIN

IV. TURBIDOMETRIC ANALYSIS OF SERUM ALBUMIN*

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(Received for publication, February 11, 1959)

The heterogeneity of serum albumin has so far been studied from many sites by using various methods such as column chromatography, differential salting out method, fractional crystalisation, turbidometric analysis, diffusion, optical rotation, electrophoresis, *etc.*

The author has investigated the turbidometric titration of protein solution, which has been applied to test the heterogeneity of serum albumin (*1-4*). In this report the author intends to deal with (i) pH dependence of turbidometric diagram of human serum albumin and (ii) variability of the subfraction of human serum albumin.

METHOD AND MATERIALS

As reported in the previous experiments (*2-4*), turbidometric titration was carried out by using ammonium sulfate solution as a precipitant and 0.16 *M* potassium phosphate buffer (0.01 *M* K-phosphate buffer, 0.15 *M* NaCl) or 0.20 *M* sodium acetate buffer (0.05 *M* Na-acetate buffer, 0.15 *M* NaCl) as a solvent. While in the previous report (*2*) protein concentration was about 0.3 per cent, in this report it was about 0.03 per cent. But both of them gave nearly the similar results but differed in some points. One example is shown in Fig. 1.

Ammonium sulfate concentration is gradually increased, and other conditions, such as pH, temperature, and concentration of protein are kept constant. Serum albumin gave a turbidity curve with a maximum at a certain concentration range of ammonium sulfate. However, at higher concentrations of ammonium sulfate, the turbidity of protein solution decreased as reported by Jirgensons (*1*) and the author (*2-4*). This phenomenon interfered the turbidometric titration. It can, however, be completely excluded by decreasing protein concentration to 0.03 ± 0.01 per cent instead of 0.3 per cent as described in the previous paper (*2*).

Human serum albumin*** was used in the experiment of pH dependence of turbidometric diagram. An electrophoretic analysis in barbital buffer at pH 8.6, ionic strength 0.1, revealed that the preparation under investigation was monodisperse,

* These data were represented at "the Symposium on the Structure of Protein" of the Japanese Biochemical Society at Kyushu University in October, 1956.

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*** Serum albumin (Cohn's Fraction V) was kindly prepared by Dr. O. Nakagawa (Institute of Nihon Seiyaku Co., Tokyo).

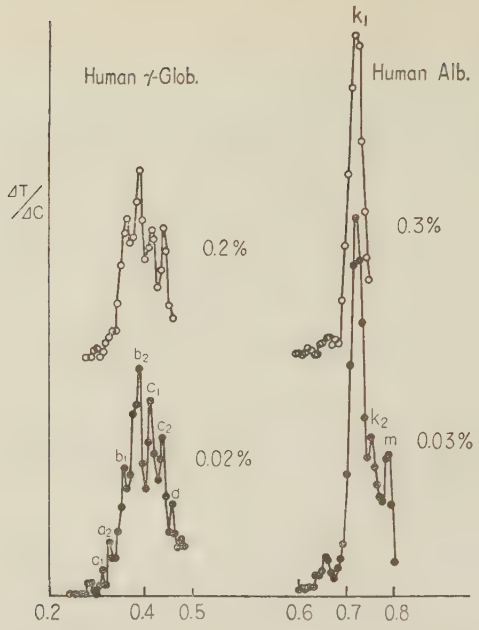

FIG. 1. Turbidometric diagram of human serum albumin and human γ-globulin, where the former being prepared by using method-B (See Table I). The abscissa and the ordinate show the volume fraction, C, of saturated ammonium sulfate solution and $\Delta T/\Delta C$, respectively, where T is relative amount of salted out protein. Experimental conditions were as follows; solvent 0.16 M phosphate buffer (10.0 ml. of 0.01 M K-phosphate buffer of pH 7.35+90 mg. NaCl), pH 6.2 at 40 per cent saturation and 5.95 at 70 per cent saturation, protein concentration γ-globulin (0.02 per cent, 0.2 per cent), human serum albumin (about 0.03 per cent, about 0.3 per cent).

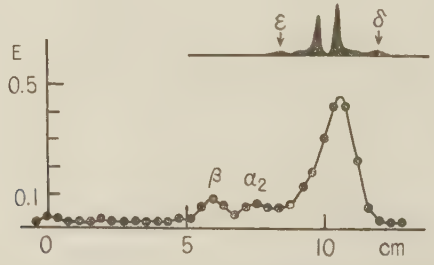

FIG. 2. An electrophoretic pattern of moving boundary method of 2 per cent human serum albumin solution in barbiturate buffer, pH 8.6, 0.1 ionic strength, and a paperelectrophoretic pattern of 3 per cent human serum albumin solution in veronal buffer pH 8.5, 0.045 ionic strength.

except showing a small amount of fraction of lower mobility. The results of moving

boundary method* and paper electrophoresis** were shown in Fig. 2. The variability of the subfraction of human serum albumin was studied with the albumin preparation by using methods A and B (Scheme 1).

SCHEME 1

The Procedure for the Preparation of Samples Used in Turbidometric Titration

Human blood

Method-A	Method-B
Standing for 10 minutes at 20~32°.	Standing for about 5 hours at 20~32.
Centrifugation for 20 minutes at 2,500 r.p.m.	Subsequent procedures are similar to method A.

Supernat. (Serum)	Precip.

10 ml. of diluted serum[1] + 10 ml. of saturated ammonium sulfate solution. Standing for 120 minutes.

Centrifugation for 30 minutes at 2,500 r.p.m.

Supernat.[2]	Precip.

1) For turbidometric analysis, serum was diluted approximately from 200 to 300 folds with K-phosphate buffer (10.0 ml. of 0.01 *M*, K-phosphate pH 7.35, +90 mg. NaCl).

2) This solution was titrated with saturated ammonium sulfate solution.

RESULTS

(1) pH Dependence of Turbidometric Diagram—pH value of protein solution was adjusted by using 0.01 *M* K-phosphate buffer or 0.05 *M* Na-acetate buffer. And then, pH value at 70 per cent saturation of $(NH_4)_2SO_4$ was given in each Figure. At higher pH regions than isoelectric point of human serum albumin, turbidometric diagram has several peaks, say, h, i, j_1, j_2, k_1, k_2, ... and then, maximum peak of the diagram shifted from h to k, i to j_1, ... according to the pH value of the solution. On the other hand, at lower pH region than isoelectric point of human serum albumin, namely at pH 3.9, turbidometric diagram showed many peaks, say, A, B. ... H, I, J. These results were shown in Fig. 3.

In evaluating the difference quotient, $\Delta T/\Delta C$, ΔC was taken on larger scale than the above mentioned one (*2*). Then, the diagram of human serum

* The author also expressed his gratitude to Dr. S. Oshima (Institute for Tuberculosis, Kyoto University, Kyoto) for his permission to use the electrophoretic apparatus.

** The apparatus of constant current was designed by Dr. H. Kiho, Dr. K. Kawai (Department of Physics, Faculty of Science, Kyoto University, Kyoto) and the author, and was constructed by the author, Dr. K. Torizuka and Prof. G. Wakizuka (Department of Internal Medicine, Faculty of Medicine, Kyoto University, Kyoto).

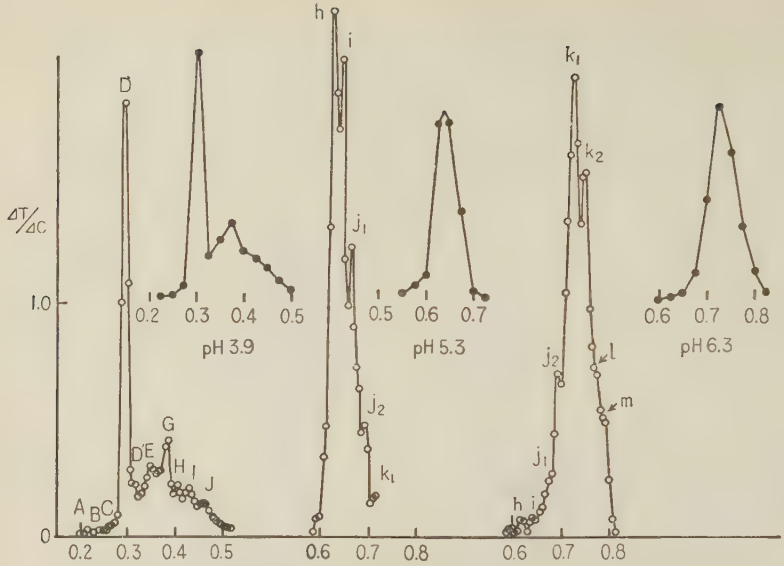

FIG. 3. pH dependence of turbidometric diagram of human serum albumin. Upper diagrams were made by smoothing out the lower diagram, *i.e.*, taking larger ΔC in upper diagrams than in lower diagrams. The abscissa and the ordinate show the volume fraction, C, of saturated ammonium sulfate solution and $\Delta T/\Delta C$, respectively, where T is relative amount of salted out protein. Experimental conditions were as follows; solvent 0.20 M acetate buffer (Na-acetate 0.05 M, NaCl 0.15 M) and 0.16 M phosphate buffer (K-phosphate 0.01 M, NaCl 0.15 M) protein concentration 0.03 per cent.

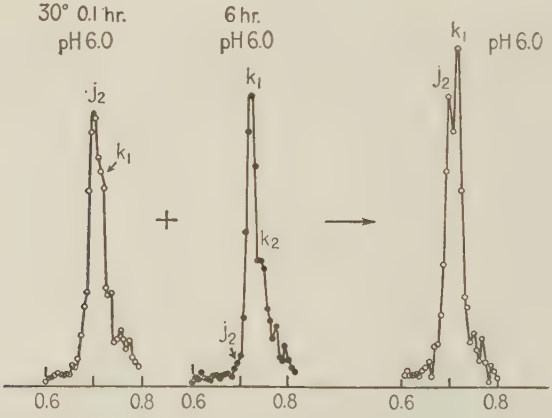

FIG. 4. Turbidometric diagram of artificial mixture of subtly different human serum albumin. Human serum albumin (j_2) and (k_1) were prepared by using methods A and B, respectively. The abscissa and the ordinate show the volume fraction, C, of saturated ammonium sulfate solution and $\Delta T/\Delta C$, respectively, where T is relative amount of salted out protein. Experimental conditions were as follows; solvent 0.16 M phosphate buffer (100 ml. 0.01 M K-phosphate buffer pH 7.35+900 mg. NaCl), pH 5.95 at 70 per cent saturation, protein concentration about 0.03 per cent.

albumin had only one peak at higher pH region and two peaks at lower pH region. These results exactly resemble the familiar electrophoretic one as shown in the upper part of Fig. 3.

(*II*) *Studies on the Variability of Subfraction of Human Serum Albumin*—At the same pH value, the turbidometric diagram of human serum albumin was markedly influenced by the ways of serum preparation (*2*, *3*, *5*). The methods used in this experiment were shown in Scheme 1. The human serum albumin prepared by method A, had its maximum at j_2, whereas one prepared by method B had its maximum at k_1. And also, when two samples were mixed together, the diagram had two maxima at j_2 and k_1. These examples were shown in Fig. 4.

DISCUSSION

The discussion will be extended in respect to the meaning of peaks in turbidometric diagram and to the comparison of the present method with the others.

(I) In the experiment of pH dependence of turbidometric diagram the following interesting facts have been shown; (a) A maximum peak shifted as pH varied, namely $h \rightarrow i$, $i \rightarrow j_1$ ... From these facts, it may be possible that the dissociation of amino acids effects on the position of main peak and on the diagram of serum albumin. (b) At the acid region than its isoelectric point, turbidometric diagram of human serum albumin had more peaks than those at pH 5.3 and 6.3. It may be due to the various kinds of N-terminal amino acids of serum albumin (*6*, *7*). (c) When the difference quotient, $\Delta T/\Delta C$ is evaluated in the turbidometric analysis, ΔC is taken on larger scale. Then, the pH dependence of turbidometric diagram resembles the familiar electrophoretic one as shown in the upper part of Fig. 3.

On the other hand, the effect of pH on electrophoretic heterogeneity was studied by many investigator (*8–19*). It was shown that the electrophoretic patterns of human serum albumin had two components at acid pH region than isoelectric point. On the other hand, Aoki and Foster (*14*, *18*) concluded from their experiment on pH dependence of electrophoretic peaks that these peaks at acid region were due to the dissociation of protein terminal groups. R. A. Phelps *et al.* (*15*, *19*) reported that these two peaks were due to an association of buffer anion with serum albumin. Recently, Keltz and Mehl (*17*), isolated a rat plasma albumin which was electrophoretically homogenous at low pH values. In the turbidometric analysis, it will be necessary to make similar experiments at acidic regions than isoelectric point.

In addition to above described facts, the subfractionation of serum albumin was carried out by many investigators at more basic region than isoelectric point of serum albumin. It was reported by using electrophoresis that serum albumin had at least two or three components at more basic regions than isoelectric point (*16*, *20*). Becker used diethylaminoethyl-cellulose for chromatographic investigation on serum albumin and demonstrated at least three components which differed in electrophoretic properties at pH 4.0, in sugar content, in

tyrosin-tryptophan ratio, and immunological behavior (*21*). Recently, by using hydroxylapatite (*22*), the author discovered that the binding ability of albumin subfractions with I^{131}-l thyroxine was very different (*23*). As stated above, serum albumin showed the heterogeneity even at higher pH region than isoelectric point. Therefore, in order to study the effect of pH on turbidometric diagram, it will be necessary to use the albumin subfraction which had almost single peak in turbidometric analysis at least at basic regions than isoelectric point*.

(II) Two kinds of human serum albumin which showed different turbidometric diagram at the same pH value could be obtained by using methods A and B (See Scheme 1). The result was shown in Fig. 4. And also, the turbidometric diagram of mixture of two kinds of human serum albumin showed the two main peaks as shown in right part of Fig. 4. The in vitro modification of serum albumin by using methods A and B did not occur at lower temperature (0–5°).

It was reported that the removal of fatty acid from serum albumin (by passage through anion exchange columns) increased dielectric increment, and that subsequent addition of sodium oleate reduced it to original value (*24*). Kendall (*27*), also obtained the two kinds of human serum albumin which were different in fatty acid content. Cohn *et al.* obtained the complex of albumin with decanol (*25*). The author studied the in vitro modification of serum albumin by using Dowex-2 anion exchanger (*26*), and obtained the three kinds of human serum albumin which showed different turbidometric diagram*.

From these data, the above mentioned change of serum albumin occurred during the isolation procedure of serum may be due to the removal of substances of small molecule.

SUMMARY

The author investigated the turbidometric diagram of serum albumin. The conclusions were as follows:

1. Turbidometric diagram of human serum albumin had more peaks at pH 3.9 than those at pH 5.3, 6.0 and 6.3.

2. It was disclosed by the turbidometric analysis that human serum albumin can easily be modified by the methods of obtaining the serum sample.

The author wishes to thank Prof. K. Sasagawa (Department of Physiology, Faculty of Medicine, Kyoto University, Kyoto) for his interest to the author's investigation. And also, the author wishes to thank Dr. M. Yamamoto (Department of Quantum chemistry, Faculty of Science, Kyoto University, Kyoto) Dr. O. Nakagawa (Nihon Seiyaku Co., Tokyo) and Dr. Y. Momotani (Department of Botany, Faculty of Science, Kyoto University, Kyoto) for their excellent advices.

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The Journal of Biochemistry, Vol. 46, No. 8, 1959

DECARBOXYLATION OF OXALOACETIC ACID BY SILK FIBROIN-PALLADIUM CATALYSTS

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(Received for publication, February 13, 1959)

Akabori and coworkers (*1-3*) have recently reported that a silk fibroin-palladium catalyst catalyses asymmetric hydrogenation of certain unsaturated compounds. The capacity of asymmetric reduction was, however, rather variable and dependent upon the nature of substrates. Thus, for instance, hydrogenation of 2-methyl-4-benzal-oxazol-5-on the presence of the silk-palladium catalyst resulted in the formation of optically active phenylalanine, but only racemic phenylalanine was obtained with α-acetamidocinnamic acid as starting material in spite of the fact that the latter reaction proceeded much easily than the former.

In an attempts to prepare optically active glutamic acid by reductive amination of α-ketoglutaric acid *at 60°* in the presence of the asymmetric catalyst, it was unexpectedly found that the main product of the reaction was succinic acid. It was therefore suspected that the catalyst may also be endowed with a capacity of decarboxylating α-keto acids. However, it was soon revealed that the catalyst was quite inactive, at least *at 30°*, in decarboxylating ordinary α-keto acids such as α-ketoglutaric, pyruvic and phenylpyruvic acids. The only exception was oxaloacetic acid which was found to be rapidly decarboxylated even at 30° in the presence of the silk fibroin palladium catalyst. The decarboxylation product was later identified as pyruvic acid. This indicates that the β-carboxyl group of oxaloacetic acid was more easily decarboxylated than the α-carboxyl one in the reaction.

This type of decarboxylation of oxaloacetic acid is of considerable interest in view of its resemblance to enzymatic reactions. The catalyst employed here is, like many enzymes, one kind of metalprotein systems and the reaction proceeds heterogenously. Furthermore, the catalysis is quite unique and differs from other decarboxylation reactions catalysed by soluble agents (*4-6*, *12*) in being remarkably specific to oxaloacetic acid. Finally, it is expected that a study of this decarboxylation reaction might provide a clue to the mechanisms underlying the asymmetric reduction which the same catalyst will be able to accomplish.

The purpose of the present paper is to describe some characteristics of the oxaloacetic acid decarboxylation catalysed by three types of silk fibroin-palladium catalysts.

MATERIALS AND METHODS

Substrates—Pyruvic (*7*), phenylpyruvic (*8*), α-ketoglutaric (*9*) and oxaloacetic acids (*10*) were prepared by the conventional synthetic methods and their purity was confirmed by analysis.

Catalysts—The following three types of catalysts were used. *Catalysts I* was prepared as follows. 100 mg. of $PdCl_2$ was dissolved in 100 ml. of 0.1 *N* acetic acid and 350 mg. of silk fibroin (was free from fatty substances) was dispersed in this solution. The suspension was then boiled for 8 minutes; the fibroin fibres gradually turning brown in color and finally precipitating as dark brown mass. The precipitate in which $PdCl_2$ is attached to the silk protein in chelate linkages, was collected, washed with water and then with methanol, and finally dried in air. *Catalysts II* was the same as employed in asymmetric reduction (*1–3*) and prepared by reducing Catalyst I with hydrogen (80 kg./cm^2) at 60° in an autoclave. In this catalyst, which is black in color, the palladium appears to be reduced to a metallic state. *Catalysts III* was prepared by repeatedly washing Catalyst I with *p*-nitrosodiethylaniline solution, a specific reagent for palladous ion (*11*). Analysis showed that no more than 50 per cent of the palladium present in Catalyst I could be eluted by this treatment. As can be expected, no palladium was removed from Catalyst II by the same treatment. These three catalysts were stable in the dry state at least for 2 months.

Decarboxylation Experiments—Decarboxylation reactions were carried out in the Warburg manometric vessels using air as the gas phase. 0.5 ml. of freshly prepared solution containing 20 μ moles of substrate (pH 5.0) was added to the side arm of the vessel. The main compartment of the vessel received 2.5 ml. of 0.1 *M* acetate buffer (pH 5.0) containing an appropriate amount (Table I) of catalyst. The vessel was equilibrated at 30° for 10 minutes, and the reaction was started by tipping. Readings were taken at 5 minute intervals.

Analytical Procedures—The palladium contents of catalysts were determined colorimetrically by the *p*-nitrosodiethylaniline method of Overholser (*11*) after careful ashing of the sample.

The decarboxylation products were detected and identified by paper chromatography of the reaction mixture before and after the 2,4-dinitro-phenylhydrazine treatment (*13*).

RESULTS

As can be seen from Table I and Fig. 1, active decarboxylation of oxaloacetic acid was observed at 30° and pH 5.0 in the presence of either Catalyst I, II or III. None of these catalysts was, however, effective in catalysing the decarboxylation of ordinary α-keto acids such as pyruvic, phenylpyruvic and α-ketoglutaric acids. The pH of medium was selected at 5.0 in order to minimize the spontaneous decarboxylation of oxaloacetic acid, but this particular pH value was fortunately found to be most favorable to the catalytic decarboxylation. This relationship is illustrated in Fig. 2. Acetate buffer was preferably used rather than the commonly used phosphate buffer because of a significant inhibition of the catalysis in the latter buffer.

Among the three types of catalysts employed, Catalyst I was the most active and Catalysts II the least. The palladium contents of these catalysts

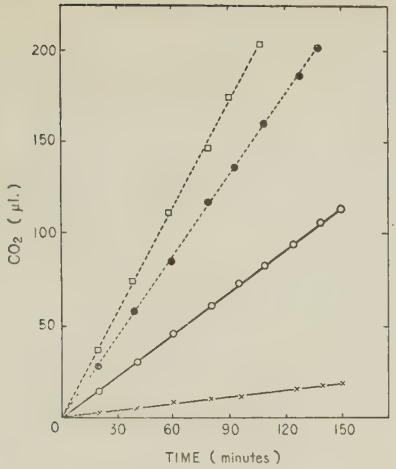

FIG. 1. Catalytic effect of Pd-silk-fibroin on decarboxylation of oxaloacetic acid.
—×— control, - -●- - catalyst (III),
—○— catalyst (II), - -□- - catalyst (I).

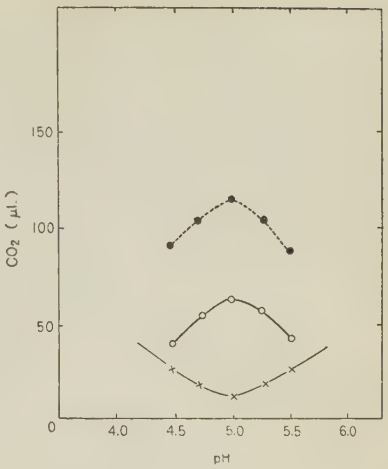

FIG. 2. Effect of pH on O. A. A. decarboxylation (at 40 min. data).
—×— spontaneous,
—○— catalyst (I),
- -●- - catalyst (III) plus NH_4Cl.

TABLE I

Catalytic Effect of Silkfibroin-Pd on Decarboxylation of OAA

	Catalyst	K*		Catalyst	K*
A	Blank	0.006	H	10 mg./3 ml. Catalyst (II) Black	0.020
B	10 μM/3 ml. NH_4Cl	0.012	I	Catalyst (III)	0.037
C	10 μM/3 ml. Glycine	0.049	J	Catalyst (I) $+NH_4Cl$	0.058
D	10 mg./3 ml. Silk fibroin	0.004	K	Catalyst (II) $+NH_4Cl$	0.030
E	Pd=900 μg./3 ml. $PdCl_2$	0.007	L	Catalyst (III) $+NH_4Cl$	0.113
F	900 μg./ml. Pb (black)	0.006	M	Glycine $+NH_4Cl$	0.052
G	10 mg./3 ml. Catalyst (I) Pd-Fibroin	0.049	N	$PdCl_2$ $+NH_4Cl$	0.012

* First-order rate constant expressed as min^{-1} $K=\frac{1.3}{t_1-t_2}\log\frac{OAA_1}{OAA_2}$ in 0.05 *M* acetate buffer pH 5.0 at 30°

recorded in Table II, on the other hand, show that Catalyst I contained the highest amount of palladium and Catalyst III the lowest. The catalytic

activity therefore seems to be independent of the palladium content, but rather to depend upon the chemical form in which the palladium is present in the catalyst. This point will be later discussed somewhat in detail. It is, however, important to know whether the association or combination of palladium with the carrier fibroin is responsible for the emergence of the catalytic activity. In fact, little or no decarboxylation of oxaloacetic acid occurred when silk fibroin , $PdCl_2$ or palladium black was separately used as the catalyst (at the concentration corresponding to the effective catalysts).

TABLE II

Palladium Content of Catalysts

	Palladium content *μg./10 mg.*	*K**
Catalyst (I)	900	0.049
Catalyst (II)	760	0.020
Catalyst (III)	490	0.037

* First-order rate constant as shown in Table I.

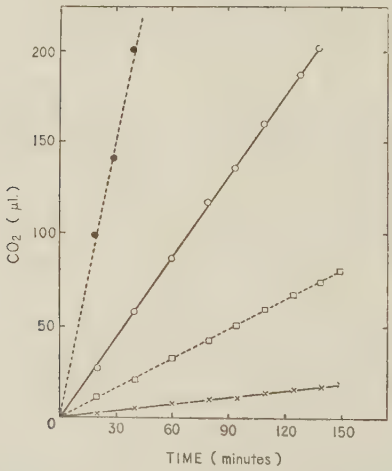

FIG. 3. Activation effect of NH_4Cl on the catalytic decarboxylation of oxaloacetic acid in 0.5 *M* acetate buffer pH 5.0 at 30°.

—×— control (20 μM/3 ml.), —○— catalyst (III), - -□- - NH_4Cl (10 μM./3 ml.), - -●- - catalyst (III)+NH_4Cl.

Bessman (*12*) reported that ammonium chloride and glycine can catalyse the decarboxylation of oxaloacetic acid. This finding was confirmed in the present investigation, but the catalytic activity of these soluble nitrogenous compounds was much lower than that of the silk-palladium catalysts. In this

connection, it is interesting to note that the decarboxylation activity of Catalyst III was appreciably increased when ammonium chloride (10 μmoles per vessel) was added to the system. Both Catalysts I and II were, however, not influenced by ammonium chloride.

Silk fibroin was found to form chelate compounds with $CuCl_2$ and $NiCl_2$ by adding these $PdCl_2$. Both silk fibroin-copper and -nickel complexes were, however, any decarboxylating activity. Furthermore, unlike the silk-palladium complex, the copper and nickel complexes were found to release easily all of the metal ions contained in them by treatment with 0.1 *N* acetic acid.

The decarboxylation product of oxaloacetic acid obtained by these types of silk-palladium catalysts was identified as pyruvic acid by paper chromatography after conversion to its 2,4-dinitrophenylhydrazone derivative. As already mentioned, pyruvic acid itself does not undergo any decarboxylation under the conditions employed. The other possible decarboxylation product, malonic semialdehyde, and its autoxidation product, malonic acid were not detected in the reaction mixture.

DISCUSSION

Two different modes of decarboxylation must be considered for oxaloacetic acid. They are:

$$HOOC \cdot CH_2 \cdot CO \cdot COOH \longrightarrow CH_3CO \cdot COOH + CO_2 \quad (1)$$

$$HOOC \cdot CH_2 \cdot CO \cdot COOH \longrightarrow OHC \cdot CH_2 \cdot COOH + CO_2 \quad (2)$$

Since the product of the reaction studied here was identified as pyruvic acid, and no traces of malonic semialdehyde and malonic acid were detected that in the reaction mixture, it is certain that the decarboxylation proceeded according to Equation 1. Consequently, this reaction is surely a β-decarboxylation.

The β-decarboxylation of oxaloacetic acid resulting in pyruvic acid is a reaction widely occurring in living cells and the enzyme is known as β-decarboxylase. It is also known that the same type of oxaloacetic acid transformation takes place spontaneously at a low rate. The various kinds of metal ions as well as amino acids and amines have been shown to promote such spontaneous β-decarboxylation of oxaloacetic acid (*12*). It was further reported that the utilization of oxaloacetic acid by liver slices can be accelerated about two-fold by the addition of glycine (*12*).

The silk-palladium compounds described in this paper differ from any of the known catalysts. They have, however, several similarities to the enzyme, socalled β-decarboxylase in point of that they are a metal-protein system and have a specificity to oxaloacetic acid, and the reaction they catalyse is heterogenous in nature. As to the heterogeneity of the reaction, it should be emphasized that these catalysts are insoluble and the palladium attached to the silk protein can not be eluted from its carrier during the reaction.

Although it is not yet possible to identify what kind of linkages of keep the palladium attached to silk fibroin in catalyst I (silk fibroin-$PdCl_2$ complex), it seems likely that at least two types of bondings are involved in the formation of the complex. One of them appears to be a rather loose linkage and readily

dissociable on treating the complex with *p*-nitrosodiethylaniline solution. The other is a strong one which can be broken only by destroying the fibroin molecule. These two types of linkages seems to exist in roughly equal amounts, since about 50 per cent of the palladium can be remove from Catalyst I by the *p*-nitrosodiethylaniline treatment. It remains to be clarified whether either one of these two types of linkages or both of them is responsible for the catalytic activity.

In Catalyst II which is a reduction product of Catalyst I, the palladium appears to be in an atomic state firmly attached to the carrier protein. Catalyst II has, though rather weak, only the decorboxylation activity despite of the fact that both palladium black and silk fibroin are quite inactive. Reasons for this are not yet clear. It might be possible that some fibroin-$PdCl_2$ chelate linkages are left unreduced even after hydrogenation of catalyst I or that the firm association of metallic palladium with silk fibroin evokes a new catalytic activity.

The fact that Catalyst III is the most effective among them is very difficult to be explained in point of its lowest palladium content. One possible explanation seems to be as follows. In Catalyst III the palladium is linked not only with the protein but also with *p*-nitrosodiethylaniline and this new type of palladium linkage is exceedingly powerful in catalysing oxaloacetic acid decarboxylation. In this connection, it is of some interest to remind that the promoting action of ammonium chloride was observed only with Catalyst III. In any way, much work is still required before the results reported here can be clearly interpreted in terms of the chemical form of palladium.

Finally, it may be of some interest to describe a possible explanation for the fact mentioned in the introduction of this paper that the formation of succinic acid from α-ketoglutaric acid during the reductive amination in the presence of the silk-palladium catalyst.

It seems probable that the succinate formation can be attributed to the thermal decomposition of a Schiff's base formed from ammonia and α-ketoglutaric acid on the surface of the palladium catalyst. In the absence of ammonia therefore, no decarboxylation of α-ketoglutaric acid should occur as was found to be the case at 30°.

SUMMARY

1. Three types of silk fibroin-palladium catalysts, *i.e.*, silk fibroin-$PdCl_2$ complex (Catalyst I), its reduction product (Catalyst II), and *p*-nitrosodiethylaniline-treated Catalyst I (Catalyst III) can catalyse active decarboxylation of oxaloacetic acid to pyruvic acid at 30° and pH 5.0.

2. Catalyst III is the most effective and Catalyst II the least, although the palladium content is highest in Catalyst I and lowest in Catalyst III. A discussion is made to interprete these results in terms of the modes of linkage between palladium and silk fibroin.

3. Some of the characteristics of the catalysed decarboxylation reaction are reported.

The author wishes to express his sincere thanks to Professor S. Akabori, Dr. R. Sato, Dr. S. Sakurai and Dr. Y. Izumi for their encouragement and advices.

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The Journal of Biochemistry, Vol. 46, No. 8, 1959

METABOLIC STUDIES OF BILE ACIDS

XXV. INTERCONVERSION OF 3-KETO AND 3-HYDROXY BILE ACIDS BY RAT LIVER EXTRACT

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(Received for publication, February 13, 1959)

Metabolic reduction of 3-keto bile acids to corresponding 3-hydroxy derivatives has long been recognized in some kinds of vertebrates (*1–3*), and the presence of an enzyme system capable of catalyzing this reaction was reasonably assumed from the results of *in vitro* studies with rat liver homogenate (*4*).

Tomkins (*5*) has recently reported on 3α-hydroxysteroid dehydrogenase obtained from mammalian source which catalyzes reversible oxidation of 3α-hydroxysteroids of both C_{19} and C_{21} series.

The present paper deals with enzymatic interconversion of 3-keto and 3-hydroxyl groups of some bile acids by rat liver extract.

EXPERIMENTAL

Preparation of Rat Liver Extract—Rat liver extract was prepared and subjected to ammonium sulfate fractionation according to the method of Tomkins (*5*). The fraction obtained at 55 to 70 per cent saturation with ammonium sulfate was used after dialysis.

Preparation of Substrate Solution—An aliquot of each bile acid preparation was dissolved in a small volume of methanol, neutralized with 0.1 *N* sodium hydroxide, and then diluted with boiling water to make final concentration of 10 per cent methanol.

Incubation and Extraction of Bile Acids—Incubation was carried out under the conditions described in Tables I and II. The incubation mixture was extracted with 5 volumes of hot ethanol, and, after cooling, filtered. Ethanol was removed from the filtrate. The residual aqueous solution was acidified with dil. hydrochloric acid and extracted with ether. The ether solution obtained was washed with water and dried over anhydrous sodium sulfate, and the ether was distilled off. The residue was then purified by means of the reversed-phase chromatography of Sjövall (*6*).

Estimation of 3-Ketocholanic Acid—3-Ketocholanic acid was estimated photometrically by the modified method of Miyashita (*7*).

Paper Chromatography of Bile Acids—The method of Sjövall (*8*) was used for free bile acids, and that of Shimizu *et al.* (*9*) for the hydroxamic derivatives of bile acids.

RESULTS

Oxidation of Lithocholic acid—Fig. 1 shows an evidence for reversible DPN+*-

* Abbreviation of oxidized diphosphopyridine nucleotide.

linked oxidation of lithocholic acid. Lithocholic acid was incubated with the enzyme preparation in the medium containing DPN$^+$ and the process of the reaction was followed spectrophotometrically by reading the absorbance of DPNH* at 340 mμ.

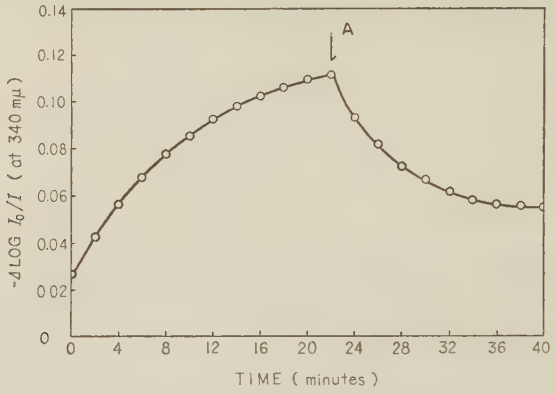

FIG. 1. Reversible oxidation of lithocholic acid.

Optical density readings at 340 mμ. The system containing 0.8 μM of DPN$^+$, 50 μM of phosphate buffer (pH 7.0), 60 μM of nicotinamide, 1.0 ml. of rat liver extract, and 0.8 μM of lithocholic acid in a total volume of 3.1 ml. Control cell contained the all components except the bile acid. Reaction started by addition of the nucleotide. At point A 0.25 μM of 3-ketocholanic acid was added. At 20°.

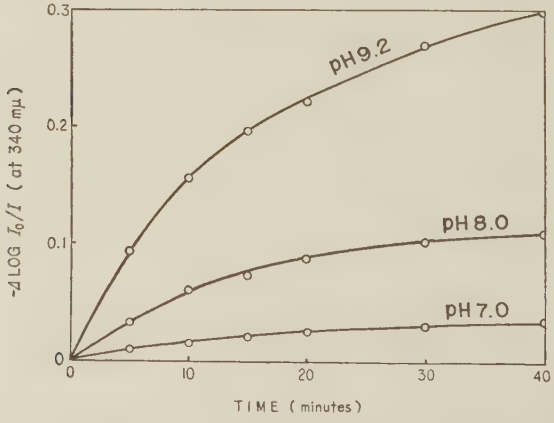

FIG. 2. Effect of pH upon the oxidation of lithocholic acid.

Spectrophotometric measurement of DPNH with timing in a 3.0 ml. system containing 0.4 μM of DPN$^+$, 50 μM of Tris buffer, 60 μM of nicotinamide, 1.0 ml. of rat liver extract, and 0.4 μM of lithocholic acid, at 22°.

* Abbreviation of reduced diphosphopyridine nucleotide.

The ascending part of the curve (Fig. 1) represents the formation of DPNH as the result of the oxidation of lithocholic acid. Reversibility of the reaction was demonstrated, when 0.25 μ mole of 3-ketocholanic acid was added at the point A in the curve, causing a decrease in optical density.

The rate of lithocholic acid oxidation was markedly dependent on pH of the medium, and it was greater at pH 9.2 than at lower pHs (Fig. 2).

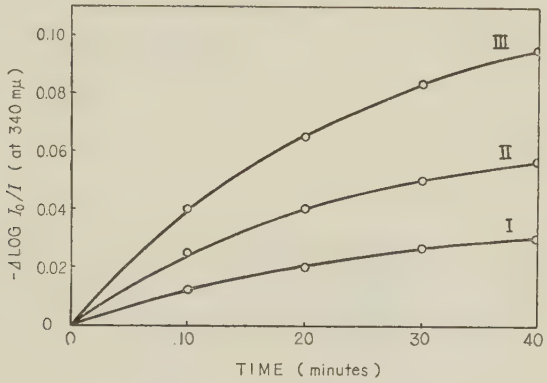

FIG. 3. Effect of the concentration of DPN^+ upon the oxidation of lithocholic acid.

Spectrophotometric measurement of DPNH with timing in a 3.0 ml. system containing 50 μM of phosphate buffer (pH 8.0), 60 μM of nicotinamide, 1.0 ml. of rat liver extract, 0.4 μM of lithocholic acid, and DPN^+ in variable amounts: 0.2 μM (curve I), 0.4 μM (curve II), and 0.6 μM (curve III), at 20°.

TABLE I

Dependence of 3-Keto Acid Formation on Amount of DPN^+

DPN^+ added (μM)	3-Keto acid formed (μM)
0	0
0.5	0.07
1.0	0.16
2.0	0.22

Incubation system contained 40 μM of phosphate buffer (pH 8.0), 40 μM of nicotinamide, 0.8 μM of lithocholic acid, 1.0 ml. of rat liver extract, and DPN^+ in a total volume of 2.5 ml. Incubated at 37° for 1.5 hours.

The rate of the reaction depended upon the concentration of DPN^+ in the medium, as demonstrated by measuring DPNH (Fig. 3), or 3-ketocholanic acid formed in the reaction mixture (Table I).

Reduction of 3-Ketocholanic Acid—The reversal of the above reaction, *i.e.* the reduction of 3-ketocholanic acid by rat liver extract was studied as follows:

As shown in Table II, the reduction reaction was coupled with the oxidation system of fructose-1,6-diphosphate in rabbit muscle extract in the presence of DPN^+ and arsenate. The rabbit muscle extract was prepared by extracting

TABLE II

Reduction of 3-Ketocholanic Acid

System	Loss of 3-ketocholanic acid	
	μM	%
Complete	0.90	75
No rat liver extract	0	0
No DPN^+	0	0

Complete system contained 20 μM of arsenate (pH 7.6), 80 μM of nicotinamide, 16 μM of cysteine, 8 μM of fructose-1,6-diphosphate, 1.2 μM of 3-ketocholanic acid, 1 μM of DPN^+, 0.5 ml. of rabbit muscle extract (see text), and 1.0 ml. of rat liver extract in a total volume of 4.0 ml. Incubated at 37° for 1.5 hours.

rabbit muscle with 0.03 *N* potassium hydroxide according to Cori *et al.* (*10*, *11*). The extract was subjected to ammonium sulfate fractionation, and the protein precipitated at 50 to 75 per cent saturation was redissolved in water, and dialyzed.

From the results obtained, a remarkable loss of 3-ketocholanic acid incubated was demonstrated in the complete system, while the rabbit muscle extract showed no reduction of 3-ketocholanic acid. The role of DPN^+ in the system was well appreciated.

Identification of the Reaction Products

1. 3-Ketocholanic Acid from Lithocholic Acid—The product of the lithocholic acid oxidation has been identified by paper chromatography. A mixture containing 5 μ moles of lithocholic acid, 50 μ moles of phosphate buffer (pH 8.0), 80 μ moles of nicotinamide, 5 μ moles of DPN^+, and 2.0 ml. of rat liver extract was incubated in a total volume of 4.0 ml. at 37° for 1 hour. Extraction of the metabolites was carried out as described above. The extract was purified by the reversed-phase chromatography (Sjövall's system (*6*)), and then subjected to paper chromatography of the two systems, the one for free bile acids (*8*), and another for the hydroxamic derivatives (*9*). Free 3-ketocholanic acid developed on paper can be located by spraying with ethanolic solution of 2,4-dinitrophenylhydrazine and the hydroxamates of bile acids on paper by spraying with ferric perchlorate.

As Fig. 4 clearly indicates, the reaction product was identified with 3-ketocholanic acid on the chromatograms of both systems. This statement was further confirmed by following experiment: The hydroxamate of the

reaction product was then boiled for 30 minutes with hydrochloric acid in ethanol (3 : 100, *v/v*) and the neutral fraction (ethyl ester) obtained from the hydrolyzate was found to give positive J a f f é ' s reaction (*7*), indicative of the presence of 3-keto group in the bile acid molecule.

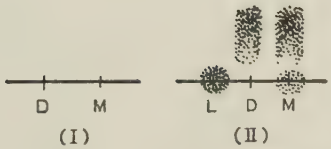

FIG. 4. Oxidation product of lithocholic acid.
(I) Chromatogram of the free acids (2,4-dinitrophenyl-hydrazine spraying).
(II) Chromatogram of the hydroxamates (ferric perchlorate spraying).
L: lithocholic acid, D: 3-ketocholanic acid,
M: metabolite.

2. Lithocholic Acid from 3-Ketocholanic Acid—In order to identify the product of the reversed reaction of the above, 50 μ moles of 3-ketocholanic acid was incubated in a medium which contained 100 μ moles of arsenate (pH 7.6), 400 μ moles of nicotinamide, 80 μ moles of cysteine, 100 μ moles of fructose-1,6-diphosphate, 5 μ moles of DPN^+, 2.0 ml. of rabbit muscle extract (see above), and 5.0 ml. of the above-mentioned rat liver extract, in a total volume of 20 ml. at 37° for 1 hour. The incubation mixture was extracted as described above, and the extract was chromatographed on the reversed phase system (*6*). By titrating each fraction of the eluate, there was obtained a band representing that of lithocholic acid (Fig. 5)

The portions of the eluate corresponding to this main band were collected, and crystalline matter was separated from them. This substance behaved exactly like lithocholic acid on the above two systems of paper chromatography (*8*, *9*) and J a f f é ' s reaction was found to be negative, indicating the absence of 3-keto group in the molecule (Fig. 6).

Although these characteristics were identical with those of lithocholic acid, digitonide formation and melting point of the free acid (m.p. 131–2°) indicated that the substance might be impure 3β-hydroxycholanic acid or rather a mixture of the stereoisomers.

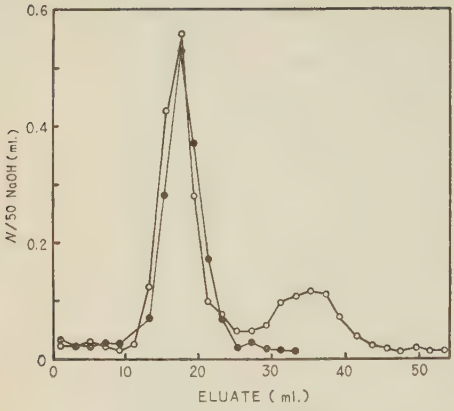

FIG. 5. Chromatogram of reaction product of 3-ketocholanic acid.

Each fraction (2 ml.) of eluate was titrated with $N/50$ NaOH. Column : 4.5 g. Hydrophobic celite. Stationary phase : chloroform/ligroin 5/5, 4 ml. Moving phase : 70 per cent methanol.

—○— reaction products,

—●— pure sample of lithocholic acid (10 mg.).

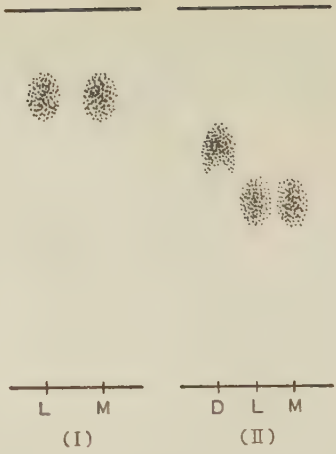

FIG. 6. Reduction product of 3-ketocholanic acid.

(I) Chromatogram of the free acids (phosphomolybdic acid spraying).

(II) Chromatogram of the hydroxamates (ferric perchlorate spraying).

L : lithocholic acid,
D : 3-ketocholanic acid,
M : metabolite.

And, furthermore, such 3β-hydroxysteroid dehydrogenase activity of the rat liver extract has been clearly illustrated by isolating iso-reductodehydrocholic acid from the incubation mixture containing 40 μ moles of dehydrocholic acid, 10 μ moles of arsenate (pH 7.6), 400 μ moles of nicotinamide, 80 μ moles of cysteine, 100 moles of fructose-1,6-diphosphate, 10 μ moles of DPN^+, 2.0 ml. of the above rabbit muscle extract, and 5.0 ml. of the rat liver extract. The incubation was carried out at 37° for 1 hour.

The ethereal extract obtained from the incubation mixture was, after being evaporated to dryness, chromatographed on the reversed phase system (*6*). From the eluate, two kinds of crystalline substance were obtained, and the one (m.p. 256°) was found to be identical with iso-reductodehydrocholic acid isolated from rabbit urine (*3*) and another with dehydrocholic acid, respectively (from their melting points and from their mixed melting points).

Relative Rates of Oxidation of Several Bile Acids—Relative rates of several bile acids were summarized in Table III. The incubation system contained 100 μ moles of sodium phosphate buffer (pH 8.0), 70 μ moles of nicotinamide, 0.4 μ mole of DPN^+, 0.4 μ mole of each of the bile acids listed, and 1.0 ml. of the rat liver extract in a total volume of 3.5 ml. In the course of the reaction (at 25°), the rate of appearance of DPNH was measured with timing (at 340 mμ). Readings were taken from 30 seconds after the addition of the rat liver extract and at every 1 minute, finally up to 5 minutes. Fig. 7 shows the oxidation rates of the individual bile acids. In order to compare the oxida-

TABLE III

Relative Rate of Bile Acid Oxidation

Bile Acid	Relative rate of oxidation
Lithocholic acid	100
Deoxycholic acid	86
Chenodeoxycholic acid	131
Hyodeoxycholic acid	110
Cholic acid	96
Glycodeoxycholic acid	90
Taurodeoxycholic acid	85
Glycocholic acid	120
Taurocholic acid	115
3α-Hydroxy-12-ketocholanic acid	114
Reducto-dehydrocholic acid	105
Glyco-3α-hydroxy-6-keto-cholanic acid	110
3α-Hydroxyallocholanic acid	93
Iso-reductodehydrocholic acid	84
Δ^5-3β-Hydroxycholenic acid	87

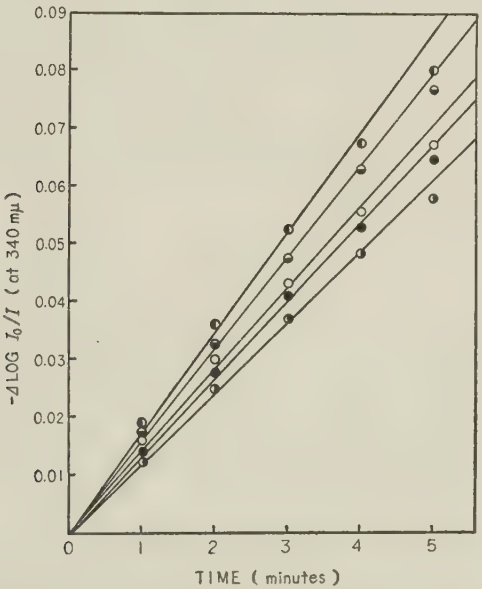

FIG. 7. Oxidation rates of some bile acids.
(The experimental condition was described in the text)
—○— lithocholic acid, —◑— deoxycholic acid,
—●— cholic acid, —◐— glycocholic acid,
—◒— taurocholic acid.

tion rate of each bile acids, the optical density increment per minute was taken from the reading of 5 minutes' intervals after the beginning of the reaction. The values in the table were expressed in per cent, that of lithocholic acid being arbitrarily taken as 100.

As shown in the table and the figure, it seems either the substitution groups at the positions other than C_3 of the cholane ring, or the conjugation of C_{24}-carboxyl group, or the steric configuration of the A-B ring junction can scarcely effect upon the reaction rate.

Relative Rates of Reduction of 3-Keto Bile Acids—The relative rates of reduction of 3-keto bile acids are indicated in Table IV. The incubation medium

TABLE IV

Ralative Rate of Reduction of 3-Keto Bile Acids

3-Keto acid	Relative rate of reduction
3-Ketocholanic acid	100
3-Ketoallocholanic acid	84
Δ^4-3-Ketocholenic acid	6

contained 100 μ moles of sodium phosphate buffer (pH 7.0), 70 μ moles of nicotinamide, 0.4 μ mole of DPN^+, 0.4 μ mole of each of the bile acids listed, and 1.0 ml. of the rat liver extract in a total volume of 3.5 ml. The activity was calculated from the initial rate of DPNH oxidation by recording the fall in the absorption at 340 mμ and the values were expressed in per cent of that of 3-ketocholanic acid.

3-Keto bile acids were reduced irrespectively of the configuration of the A-B ring junction, while the presence of α,β-unsaturation in the molecule remarkably prevented the reaction.

DISCUSSION

The presence of the enzyme system in vertebrates which catalyzes the conversion of 3-keto group of some bile acids to 3α- and/or 3β-hydroxyl groups has long been inferred from the results of *in vivo* studies (*1–3*).

Hayaishi *et al.* (*12*) have reported on a DPN^+-linked dehydrogenase obtained from bacterial source, which catalyzes the reversible dehydrogenation of 3α-hydroxycholanic acid and also shows an activity of 3β-hydroxysteroid dehydrogenase.

From the data presented here, it has become clear that the rat liver contains an enzyme system (or systems), capable of catalyzing the reversible DPN^+-linked dehydrogenation of both 3α- and 3β-hydroxycholanic derivatives. But it is not clear at present whether a single enzyme (or enzyme system) is responsible for dehydrogenation of both 3α- and 3β-hydroxycholanic acids or not, though recently Yamasaki *et al.* (*13*) were able to obtain from the rat liver a β-hydroxysterol dehydrogenase preparation which was devoid of α-

hydroxysteroid dehydrogenase activity.

SUMMARY

1. Reversible DPN^+-linked dehydrogenation of bile acids catalyzed by rat liver extract was described.

2. The extract has an activity of dehydrogenating both 3α- and 3β-hydroxyl groups of bile acids.

3. It seems likely that the reaction can proceed irrespectively of the substitution groups in the cholane skeleton, and the conjugation of carboxyl group, as well as of the steric configuration of the A-B ring junction.

The author expresses his hearty thanks to Prof. Dr. K. Yamasaki for his kind guidance throughout this research.

The present work was partly aided by the Grant in Aid for Scientific Research from the Ministry of Education.

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METABOLIC STUDIES OF BILE ACIDS

XXVI. OXIDATION OF Δ^5-3β-HYDROXYCHOLENATE IN SOME TISSUE HOMOGENATES

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(Received for publication, February 13, 1959)

Ogura *et al.* (*1*) have reported certain aspects of 3-keto-bile acid metabolism in rat liver homogenate and proposed a metabolic pathway from Δ^4-3-ketocholenic acid to lithocholic acid through 3-ketocholanic acid. Though Δ^5-3β-hydroxycholenic acid might not be assumed at present as an intrinsic metabolite from cholesterol to bile acids (*2*) or to steroid hormones, it is an available model compound for the metabolic studies of cholesterol, because it has the same nuclear structure as cholesterol has.

In this paper, enzymic oxidation of Δ^5-3β-hydroxycholenic acid to Δ^4-3-ketocholenic acid by some mammalian tissue homogenates is reported.

EXPERIMENTALS

Preparation of Δ^5-3β-Hydroxycholenic Acid—This compound was prepared from hyodeoxycholic acid according to the method described by Yamasaki and Ushizawa (*3*).

Preparation of Δ^5-3β,7α(?)-Dihydroxycholenic Acid (m.p. 214–5°)—This diol was prepared from methyl Δ^5-3β-acetoxy-7-ketocholenate (*4*) by potassium borohydride reduction. It will be reported elsewhere.

Preparation and Fractionation of the Tissue Homogenates—All manipulations of tissues were carried out at 0–5° until the homogenate or its fractions were ready for incubation. Each of the tissues was ground for 1 minute in a glass homogenizer either with 0.9 per cent potassium chloride solution or with 0.25 *M* sucrose solution, diluted with the homogenizing fluid to give a 10 per cent homogenate of the adrenal, or 20 per cent homogenate of the other tissues.

Preparation of the mitochondrial and microsomal parts from adrenal homogenate was carried out by the slightly modified technique of Schneider and Hogeboom (*5*). The homogenate was precipitated at 600×*g* for 10 minutes and the supernatant was again centrifuged at 8,500×*g* for 10 minutes, the sediment containing the mitochondrial part was finally obtained. This fraction was suspended in 0.25 *M* sucrose solution by means of a glass homogenizer.

The microsomal suspension was likewise prepared from the particles spun down at 8,500×*g* for 10 minutes—80,000×*g* for 60 minutes.

Incubation Procedure—0.5 ml. of the homogenate or of each fraction of it was transferred to a tube containing 0.6 μ mole of sodium Δ^5-3β-hydroxycholenate dissolved in 0.1 ml.

of propylene glycol-water (1 : 1 *v/v*), 0.1 ml. of 0.2 *M* potassium phosphate buffer (pH 7.4), 0.1 ml. of 0.2 *M* nicotinamide, 0.1 ml. of 0.02 *M* diphosphopyridine nucleotide (DPN^+) and 0.1 ml. of water. The tubes were placed in a bath of 37° with mechanical shaking for 1 hour.

Extraction Procedure—Five volumes of methanol were added to one volume of the incubation medium, and the precipitate formed was centrifuged; this was suspended in five volumes of methanol and again centrifuged. These methanol extracts were combined and concentrated until methanol was almost completely driven off. The aqueous extract was made alkaline by addition of five volumes of 2 per cent sodium carbonate solution and extracted twice with ether to eliminate lipid substances. The aqueous layer was acidified (congo red) by addition of 10 per cent hydrochloric acid, and again extracted twice with ether. The combined ether extract was washed with water, dried over sodium sulfate, and evaporated to dryness. The residue was chromatographed as described below.

Chromatographic Procedure—The reversed-phase partition chromatography described by Bergström and Sjövall (*6*, *7*) was used for separation of the metabolites. Two ml. of 65 per cent (*v/v*) chloroform-heptane mixture supported on 2.5 g. of hydrophobic Supercel was used as stationary phase and 60 per cent (*v/v*) aqueous methanol as moving phase.

Estimation of Δ^4-3-Ketocholenic, Δ^5-3β-Hydroxycholenic and Δ^5-3β,7α(?)-Dihydroxycholenic Acids—The amount of Δ^4-3-ketocholenic acid was determined by estimation of the ultraviolet absorbance (at 240 mμ) which is characteristic of α,β-unsaturated ketone.

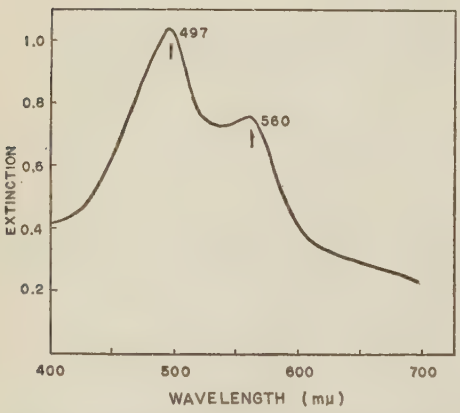

FIG. 1. Absorption spectrum of the colored substance obtained from Δ^5-3β-hydroxycholenic acid treating with the Kiliani reagent.

FIG. 2. Calibration curve of the colored substance obtained from Δ^5-3β-hydroxycholenic acid in the Kiliani reaction.

The Kiliani reaction (*8*), which is used for determination of serum cholesterol, was found to be applicable for determination of Δ^5-3β-hydroxycholenic acid by estimation of the intensity of its violet color as will be reported elsewhere (*9*). The absorption

spectrum of the reaction product shows a strong maximum at 497 mμ with a weak peak at 560 mμ (Fig. 1). To 3.0 ml. of glacial acetic acid solution containing 20–300 μg. of Δ^5-3β-hydroxycholenic acid was added 2.5 ml. of a freshly prepared mixture of 100 parts of concentrated sulfuric acid and 1 part of 10 g./dl. ferric chloride solution in glacial acetic acid. The mixture was shaken well and allowed to stand for 20 minutes at room temperature; its optical density was then determined spectrophotometrically against a blank. Beer's law was obeyed at the concentrations ranging from 50 to 300 μg. per 3 ml. of acetic acid (Fig. 2). Each chromatographed fraction of the incubation mixture was freed from the solvent, dried and then subjected to this determination.

Δ^5-3β, 7α(?)-Dihydroxycholenic acid was estimated by the Lifschütz reaction devised for the determination of 7α-hydroxycholesterol, as described by Yamasaki *et al.* (*12*).

RESULTS

I. Oxidation of Δ^5-3β-Hydroxycholenate in Adrenal Homogenate—Three μ moles of sodium Δ^5-3β-hydroxycholenate was incubated with rabbit adrenal homogenate in 5 ml. of the incubation medium as mentioned above, and the extract obtained from the medium was subjected to the reversed-phase partition chromatography, the effluents being estimated either spectrophotometrically (at 240 mμ) or by the Kiliani method.

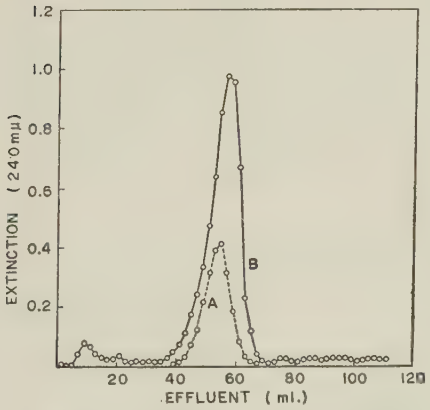

FIG. 3. Reversed phase partition chromatography of pure Δ^4-3-ketocholenic acid (curve A) and that of the extract from the incubation medium of Δ^5-3β-hydroxycholenate with rabbit adrenal homogenate.

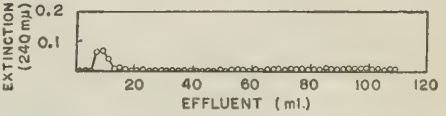

FIG. 4. Reversed-phase partition chromatography of the extract obtained from the blank test.

(i) The extract, when spectrophotometrically estimated, showed two bands on the chromatogram, the first minor band appearing in 10 ml.-effluent region and the second main band in 58 ml.-region. As shown in Fig. 3, the main band on the chromatogram coincided with that of Δ^4-3-ketocholenic acid. The

total amount appearing at this band (Δ^4-3-ketocholenic acid) was 1.9 μ moles, which indicates that 64 per cent of the added Δ^5-3β-hydroxycholenate was converted into Δ^4-3-ketocholenate. Ultraviolt absorption curve of this fraction coincided fairly well with that of Δ^4-3-ketocholenic acid in aqueous methanol in respect to their maximum absorption at 245 mμ. (Fig. 5).

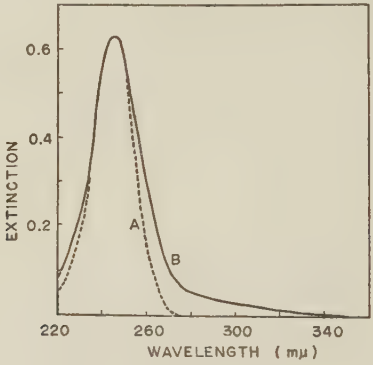

FIG. 5. Ultraviolet absorption spectra of pure Δ^4-3-ketocholenic acid (curve A) and that of the extract obtained from the incubation medium (curve B).

The first minor band mentioned above was also found to appear, in the same manner both in localization and in quantity as that on the chromatogram of the extract obtained from the blank test (Fig. 4), so that the compound responsible for this band is not any metabolite of Δ^5-3β-hydroxycholenate, but for some acidic contaminant in the adrenal homogenate.

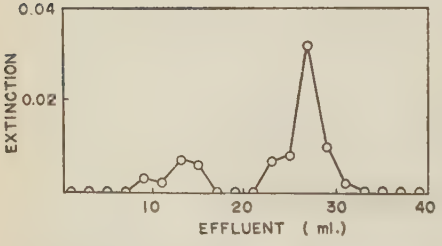

FIG. 6. Reversed-phase partition chromatography of the extract obtained from the incubation medium, the effluents being estimated by the Kiliani reaction.

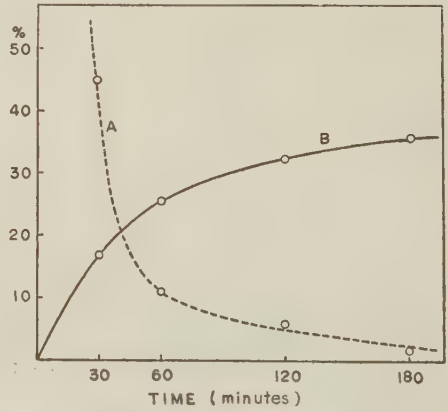

FIG. 7. The rate of decrease of Δ^5-3β-hydroxycholenic acid (curve A) and formation of Δ^4-3-ketocholenic acid (curve B) on the incubation of Δ^5-3β-hydroxycholenic acid with rabbit adrenal homogenate.

(ii) Fig. 6 presents the chromatogram of the extract, the effluents being estimated by the Kiliani reaction, and as shown in the figure, only a small portion of the substrate (0.04 μ mole; recovery 0.13 per cent) was recovered unchanged (in 24 to 32 ml.-effluent regious).

From the experimental data described above, it becomes evident that Δ^5-3β-hydroxycholenate is metabolized in the adrenal homogenate to Δ^4-3-ketocholenate. This statement is further confirmed by the following experiment: Δ^5-3β-hydroxycholenate was incubated with the adrenal homogenate as described above, and both the substrate and its metabolite, Δ^4-3-ketocholenate, were simultaneously determined as the incubation time proceeded. As clearly demonstrated in Fig. 7, the increase of Δ^4-3-ketocholenate was exactly in proportion to the disappearance of the substrate in the medium.

Identification of the Metabolite, Δ^4-3-Ketocholenate—In order to identify the metabolic product, 20 mg. of Δ^5-3β-hydroxycholenic acid was incubated under the same condition as described above, and the ether-soluble acidic fraction was subjected to the reversed-phase chromatography, as mentioned above.

Several portions corresponding to Δ^4-3-ketocholenic acid were combined; most of the solvent (methanol) was driven off on a water bath; the remaining aqueous solution was acidified with dil. hydrochloric acid and extracted three

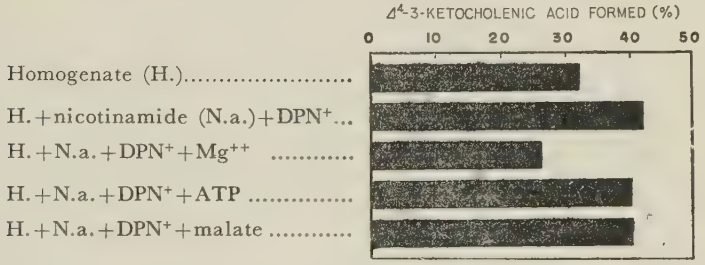

FIG. 8. Influence of some co-factors upon enzymic dehydrogenation of Δ^5-3β-hydroxycholenic acid. Concentration of co-factors: 20 μM nicotinamide, 2 μM DPN$^+$, 10 μM $MgCl_2$, 2 μM ATP and 10 μM malate in 1 ml.

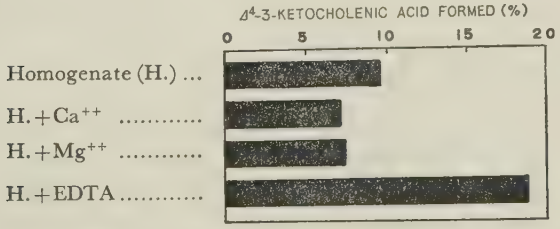

FIG. 9. Influence of calcium and magnesium ions, and of EDTA upon enzymic dehydrogenation of Δ^5-3β-hydroxycholenic acid. Each concentration of $CaCl_2$, $MgCl_2$ and EDTA was 0.01 *M*.

times with ether. The combined ether solution was washed with water until it was free from hydrochloric acid and then evaporated to dryness. The

residue was dissolved in 4 ml. of ether-petroleum ether (b.p. 35–60°) mixture and left at room temperature until it was completely crystallized. One recrystallization from the same solvent mixture yielded 2 mg. of crystals melting at 186°, undepressed on admixture with authentic Δ^4-3-ketocholenic acid (m. p. 187°). The crystal was methylated with diazomethane as usual, and the resulting ester melted at 125°, exactly the same as methyl Δ^4-3-ketocholenate.

The Effect of Some Co-factors—As shown in Fig. 8, the addition of DPN^+ and nicotinamide to the incubation medium enhanced remarkably the enzymic activity while no appreciable effect was observed in experiments, in which adenosine triphosphate (ATP) or sodium malate was further added to the system. On the other hand, the addition of magnesium or calcium ion caused an inhibition of the enzymic activity (Fig. 9), and there was found almost a doubling of the yield of Δ^4-3-ketocholenic acid formed when 20 μ moles of ethylenediamine tetraacetate (EDTA) was added.

Effect of pH—Incubations were carried out in media of several pHs, from pH 5.8 to pH 9.0 (0.1 *M* phosphate buffer being used for pH 5.8–7.4, and 0.1 *M* tris-(hydroxymethyl)-aminomethane buffer, for 8.0–9.0). As Fig. 10 shows, dehydrogenation of Δ^5-3β-hydroxycholenic acid was enhanced as the pH was increased, so far as the pHs were tested.

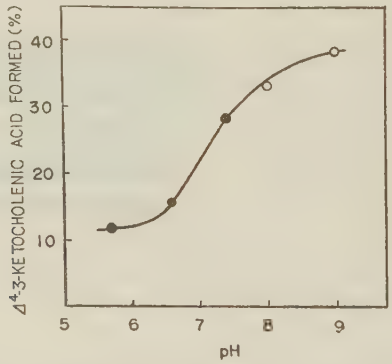

FIG. 10. Effect of pH on enzymic dehydrogenation of Δ^5-3β-hydroxycholenic acid.
● phosphate buffer, ○ tris buffer.

Distribution of the Enzymic Activity in the Adrenal Homogenate—As shown in Table I, the microsomal fraction showed the highest enzymic activity among the fractions of the homogenate. The activity in the mitochondrial fraction was decreased by repeated washings with 0.25 *M* sucrose solution at 8,500×*g* for 10 minutes, while the enzymic activity in the soluble fraction was not decreased even by further centrifugation at 80,000×*g* for 2 hours.

Enzymic Activity in Acetone-Powdered Adrenal Gland—One g. of rabbit adrenal glands was homogenized with 20 ml. of cold acetone (-15°), and the precipitate was washed with 10 ml. of the same solvent. The precipitate, dried in reduced

TABLE I

Distribution of Enzymic Activity among the Fractions of Adrenal Homogenate

Fraction	Δ^4-3-Ketocholenic acid formed	
	μM	%
$600\times g$ supernatant	0.300	50.2
Washed mitochondria	0.055	9.2
Microsome	0.120	20.0
$80{,}000\times g$ supernatant	0.071	11.8

TABLE II

Enzymic Activity in Acetone-Powdered Adrenal Gland of Rabbit

Medium	Δ^4-3-Ketocholenate formed	
	μM	%
Complete system	0.126	21.0
,, +pyruvate	0.147	24.5

pressure, was extracted with 6.0 ml. of 0.1 *M* potassium phosphate buffer (pH 7.4), centrifuged at $10{,}000\times g$ for 30 minutes, and the clear supernatant, red in color, was used as an enzyme preparation. With this enzyme preparation, 0.6 μ mole of Δ^5-3β-hydroxycholenate was incubated with the enzyme preparation and an increase of ultraviolet absorption at 240 mμ, corresponding to 0.126 μ mole (yield: 21 per cent) of Δ^4-3-ketocholenic acid, was found in the

TABLE III

Metabolism of Δ^5-3β-Hydroxycholenate and Δ^5-3β,7α-Dihydroxycholenate by the Particle-free Supernatant of Rat Liver Homogenate

Each incubation medium contains 0.5 ml. of supernatant, 2 μM DPN$^+$, 20 μM nicotinamide and 10 μM $MgSO_4$.

Incubation medium	Substrate (μM)	Incubation time (*min.*)	Substrate disappeared	
			μM	%
Complete	Δ^5-3β-hydroxycholenate, 0.25	30	0.085	34.8
,,	,, ,,	60	0.098	39.2
,,	,, ,,	120	0.104	41.6
Without DPN$^+$	,, ,,	120	0.011	4.4
Complete	Δ^5-3β,7α-dihydroxycholenate, 0.25	120	0.057	22.8
Without DPN$^+$	,, ,,	120	0.000	0.0

ether extract of the incubation medium. The ultraviolet absorption spectrum of the extract was completely coincident with that of Δ^4-3-ketocholenic acid. The enzymic activity was slightly accelerated by further addition of 20 μ moles of sodium pyruvate to the incubation medium (Table II).

II. Oxidation of Δ^5-3β-Hydroxycholenate in Other Tissue Homogenates—Further experiments similar to the above were carried out with liver, kidney and spleen homogenates of rat. Under the same condition, as in the experiment with the adrenal homogenate, Δ^5-3β-hydroxycholenate was incubated with these homogenates, and the amount of Δ^4-3-ketocholenic acid then formed in each case was found to be within the limit of 2 per cent error. Remarkable disappearance of Δ^5-3β-hydroxycholenate as well as Δ^5-3β,7α-dihydroxycholenate, however, was proved when the particle-free supernatant of rat liver homogenate was used as shown in Table III.

DISCUSSION

Samuels *et al.* (*10*, *11*) reported that enzymic oxidation of Δ^5-3β-hydroxysteroids to Δ^4-ketosteroids was demonstrable in some mammalian tissues, especially, in the endocrine organs that will excrete non-bezenoid steroid hormones. According to these authors, the enzyme or enzyme system responsible for the reaction required DPN^+ as a cofactor, and the microsomal fraction had the highest enzymic activity among the adrenal cell fractions. The present experiment showed that Δ^5-3β-hydroxycholenate was also dehydrogenated by the same fraction of adrenal homogenate of rabbit as described by Samuels *et al.*, namely, by steroid-3β-ol-dehydrogenase.

It is well known that adrenal tissue contains some other steroid-metabolizing enzymes, *e.g.* the enzymes oxigenating at the C_{21} or C_{11} position of the steroid molecule. On the chromatogram of the metabolic products in the present experiment, any detectable absorption in ultraviolet region, especially at 240 mμ, was not demonstrated in the fractions more polar than that of Δ^4-3-ketocholenate, as shown in Fig. 3, so that a further oxidation of Δ^4-3-ketocholenic acid formed would be negligible under the condition adopted here.

The fact that calcium or magnesium ion strongly inhibited the enzymic action could be explained as follows: solubility of the alkaline earth salts of Δ^5-3β-hydroxycholenate is too low to be acted on. Consequently EDTA, removing magnesium or calcium ion from the reaction medium, assuredly accelerated the enzymic activity.

The particle-free supernatant of rat liver homogenate did show an appreciable activity of metabolizing Δ^5-3β-hydroxycholenate, while the formation of Δ^4-3-ketocholenic acid from the latter in the tissue homogenates other than that of the adrenal gland could not be proved in our experiments. Recently Yamasaki *et al.* (*12*) have demonstrated the existence of 3β-hydroxysterol dehydrogenase in a particle-free supernatant of rat liver homogenate, which was able to oxidize not only Δ^5-3β-hydroxysterols, but also Δ^5-3β-hydroxycholenate. The present experiments also confirmed this finding (Table III).

SUMMARY

1. Δ^4-3-Ketocholenic acid has been identified as a metabolite of Δ^5-3β-hydroxycholenic acid in adrenal homogenate of rabbits.

2. It was found that DPN^+ was required as a co-factor, and, that both calcium and magnesium ions inhibited the enzymic activity, while ethylenediamine tetraacetate remarkably enhanced the reaction.

3. The microsomal fraction of adrenal homogenate contained the highest enzymic activity among the fractions obtained therefrom.

The author expresses his hearty thanks to Prof. Dr. K. Yamasaki for his kind guidance throughout the research.

The present experiment was supported partly by the Grant in Aid for the Scientific Research from the Ministry of Education.

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The Journal of Biochemistry, Vol. 46, No. 8, 1959

METABOLISM OF ANTHRAQUINONE

II. SULFATE CONJUGATE OF 2-HYDROXYANTHRAQUINONE

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(Received for publication, March 11, 1959)

In a previous study anthraquinone was fed to rats and 2-hydroxyanthraquinone was recovered from the urines (*1*). However, its quantity was found to be very small when freshly voided urine was examined by paper chromatography. This fact suggested that the urine contained a substance which liberated 2-hydroxyanthraquinone on standing. Using S^{35}-sulfate a sulfate conjugate of 2-hydroxyanthraquinone was found.

EXPERIMENTAL AND RESULTS

Rats were fed with anthraquinone in the same way as reported previously (*1*). About 100 μc. of radioactive sulfate was injected subcutaneously into the rat and the urine excreted within the next 24 hour period was collected.

Radioautography of the Chromatograms—Ascending chromatography was carried out on Whatman No. 1 paper and the paper was subjected to radioautography. There appeared a spot with R_f value of 0.51 and 0.39 when run in butanol, acetic acid, water mixture (4 : 1 : 1), and in 3 *N* Na_2CO_3, 3 *N* NH_4OH, and butanol (3 : 3 : 9), respectively.

Colour Reaction of the Substance on the Chromatogram—The colour of this spot on the paper was pale yellow and gave no fluorescence under ultraviolet light. The colour did not change by spraying 10 per cent Na_2CO_3 solution followed by diazotized sulfanilic acid. But after the paper was placed in an atmosphere of hydrogen chloride fume for 20 minutes at room temperature, the spot was coloured yellow and turned to red by spraying with alkali. This colour reaction of the hydrolyzed material was the same as that of 2-hydroxyanthraquinone.

The quantity of 2-hydroxyanthraquinone which was contained in this spot was not enough to account for all the concentration of 2-hydroxyanthraquinone found in the urine.

Examination of the Acid Hydrolyzed Product of the Spot—The radioactive spot

was cut out from the paper and eluted with water. Then the eluate was acidified with hydrogen chloride solution to a concentration of 1 *N* and heated at 100° for 1 hour. The solution was condensed by evaporation to a small volume and submitted to paper chromatography using different solvents as described in the previous report (*1*). The R_f values of the obtained spot were in good agreement with those of 2-hydroxyanthraquinone. The spot was cut out and eluted with alcohol. The absorption curve from 210 mμ to 600 mμ coincided with the authentic sample of 2-hydroxyanthraquinon (*1*). Inorganic S^{35}-sulfate was also detected, but no other product was found by fluorescence or colour reaction such as spraying with alkali or diazo reagents.

Stability of the Compound—This compound was labil in water and yielded 2-hydroxyanthraquinone on standing.

An attempt was made to recover the sulfate conjugate in a considerable quantity from rat urine by counter current technique using water and butanol as solvents; but the compound was almost completely decomposed during the manipulation.

Synthesis of the Sulfate Conjugate of 2-Hydroxyanthraquinone—10 mg. of 2-hydroxyanthraquinone was dissolved in 0.75 ml. of pyridine at 0°. To this was added slowly a mixture of 0.05 ml. of chlorosulfonic acid and 0.125 ml. of chloroform. The mixture was stirred for one hour and centrifuged. The supernatant was neutralized with potassium hydroxide solution and subjected to paper chromatography which showed a spot in the same location and with the same reactions and components as were found in the urine of rats fed with anthraquinone. Further purification was abandaned because it was decomposed easily by further manipulation.

Attempts were made, but without success, to conjugate S^{35}-sulfate with 2-hydroxyanthraquinone in rat liver slices or in the supernatant of rat liver homogenates by the method described before (*2*).

DISCUSSION

Rats were fed with anthraquinone and a metabolite which decomposed to sulfate and 2-hydroxyanthraquinone was found in the urine. By paper chromatography and colour reactions this substance was found to be the same with a compound recovered from a procedure to conjugate sulfate with 2-hydroxyanthraquinone. From this evidence the metabolite was concluded to be the sulfate conjugate of 2-hydroxyanthraquinone.

SUMMARY

Urine from rats fed anthraquinone and S^{35}-sulfate was examined by paper chromatography and colour reactions. A metabolite which decomposed tos ulfate and 2-hydroxyanthraquinone was found, and it was concluded to be a sulfate conjugate of 2-hydroxyanthraquinone.

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The Journal of Biochemistry, Vol. 46, No. 8, 1959

ON THE AXIAL RATIO OF THE SUBUNIT OF CATALASE MOLECULE

A CORRECTION TO THE PREVIOUS REPORT

In a recent publication (Samejima T., *J. Biochem.*, 46, 155 (1959)), it was reported that the catalase molecule can be split by alkaline treatment (pH= 12.0) into smaller homogeneous components, having a molecular weight of 85,000 and an axial ratio of 1.8, which were stated to be approximately one third of those found for native catalase, respectively.

Owing to an inadvertent mistake made in the calculation, a correction should be made of the value of axial ratio of the split subunit. The corrected value is 26, which is 5 times that of native catalase. Since the molecular weight of the subunit is one third of that of native catalase, there is no doubt that native catalase is made up of three subunits. However, one of the conclusions made in the previous report that the subunits are connected lengthwise in native catalase to form a rod-shaped molecule, loses its ground in view of the revised calculation.

Thanks are due to Prof. K. Shibata for calling my attention to this miscalculation.

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(Received for publication, June 18, 1959)

昭和 32 年 7 月 9 日 第三種郵便物認可
第 46 巻 第 8 号 昭和 34 年 8 月 25 日 発行
(毎月 1 回 25 日発行)
The Journal of Biochemistry, published monthly by the Japanese Biochemical Society, Tokyo, Japan.
Vol. 46, No. 8, August 25, 1959
Vol. 46 **No. 8**

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